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Implications

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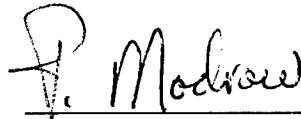
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FOREWORD

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5. INTRODUCTION

A number of human breast cancers manufacture β -adrenergic receptors, for which there are drugs available that stimulate or inhibit their activity. The current study investigates the regulation and function of these receptors and identifies factors that can promote the ability of the receptors to influence cell division. In normal cells, the receptors are linked to the genes that control cell division only during a discrete developmental period in which they first enhance, and then terminate cell replication. We hypothesize that the redifferentiation that accompanies carcinogenesis will render cells responsive in the same way as are developing cells, but without the subsequent loss of reactivity that normally occurs as cells mature. There are three Specific Aims: (1) to determine whether β -receptors are linked to protooncogene expression and to evaluate the ability of the receptors to downregulate or desensitize upon exposure to a stimulant (isoproterenol); (2) to determine whether factors that promote the activity of β -receptor signaling lead to altered protooncogene expression and inhibition of cell replication — inhibitors cAMP breakdown (theophylline), or glucocorticoids (dexamethasone); (3) to determine whether stimulation or blockade of β -receptors can be used to control cell replication. In extending these results to detection and novel treatment paradigms, biopsy can readily identify the presence of β -receptors on breast cancer cells, indicating patients in whom restriction of caffeinated beverages and β -blocker therapy may serve as a useful addition to surgical, chemotherapeutic and radiation interventions. Perhaps most importantly, because β -adrenergic control of protooncogene expression is “upstream” from cell cycle control, interventions aimed at receptor-driven events should still operate even after multidrug resistance appears.

6. BODY

Relationship of progress to each task described in the original Statement of Work:

The original Statement of Work contained the following tasks:

Task 1 — Dose-response and time-response curves for isoproterenol sensitization or desensitization of β -receptors and adenylyl cyclase

Task 2 — Dose-response and time-response curves for isoproterenol induction of *c-fos* protooncogene expression

Task 3 — Dose-response and time-response curves for isoproterenol effects on DNA synthesis and cell number

Task 4 — Dose-response and time-response curves for isoproterenol effects on nuclear labeling

Task 5 — Dose-response and time-response curves for isoproterenol sensitization or desensitization of β -receptors and adenylyl cyclase in the presence or absence of theophylline; and in the presence or absence of dexamethasone — contains all the elements of Tasks 1, 2, 3

Task 6 — Ability of propranolol to block stimulatory effects of isoproterenol — contains all the elements of Tasks 1, 2, 3

For practical reasons, we chose to concentrate first on the demonstration that isoproterenol treatment could indeed interfere with breast cancer cell replication. We switched from the originally-proposed cell line (CG-5) to another line (MDA-MB-231) when it became apparent that the latter actually had a much higher concentration of β -adrenoceptors, was more readily available, and had a more demonstrable, widespread distribution in human cancers. To remain within a unitary set of techniques for each phase of the study, our first year concentrated on cell replication,

cell number, adenylyl cyclase and receptor regulation as the targets (Task 1, Task 3, and the corresponding sections of Tasks 5 and 6). At this stage, we are actually slightly ahead of the projected schedule, as we have completed 60 weeks of the projected timetable within the span of 52 weeks. In year 2, we will concentrate on protooncogene expression (Task 2) and its corresponding parts in Tasks 5 and 6. Year 3 is projected to concentrate on Task 4 and its corresponding sections of Tasks 5 and 6.

The body of this report is divided into three sections: and Introduction to the problem, Methods, and Results. Discussion of the results obtained, their importance and implications, appear in Section 9 — Conclusions.

Introduction

In addition to their role as neurotransmitters and “stress” hormones, catecholamines play a trophic role in the control of cell replication and differentiation in target cells that express adrenergic receptors. Lower organisms, such as sea urchins, overexpress norepinephrine, epinephrine and other biogenic amines during critical developmental periods in which these amines control cell replication and differentiation (Buznikov et al., 1970). In mammals, “spikes” of adrenergic activity also modulate the rate of cell replication and differentiation and thus control the architectural modeling of adrenergic target tissues (Vernadakis and Gibson, 1974; Claycomb, 1976; Lovell, 1982; Slotkin et al., 1987, 1988a). The importance of adrenergic control of mammalian cell development has recently been pointed out by the lethal effects of gene knockouts that eliminate the ability to synthesize norepinephrine or to express β -receptors; these animals die *in utero* from disruption of cardiac cell replication/differentiation and consequent dysmorphogenesis (Thomas et al., 1995; Zhou et al., 1995; Rohrer et al., 1996). The critical period for adrenergic control of these events terminates as cells exit mitosis and approach terminal differentiation, so that the sensitivity to adrenergic stimulation of cell replication disappears in adulthood except for a few tissues that undergo continual renewal (Claycomb, 1976; Slotkin et al., 1987; Wagner et al., 1994; Zeng et al., 1996).

It is thus of critical importance that, with carcinogenic redifferentiation, many cell types, including epithelial cancers and cancers of secretory cells, re-express β -adrenergic receptors (Ling et al., 1992; MacEwan and Milligan, 1996; Re et al., 1996; Canova et al., 1997; Mitra and Carraway, 1999), which can once again resume their role in the control of cell replication (Re et al., 1992, 1996; Mitra and Carraway, 1999). In some cell lines, β -adrenergic stimulation elicits a small, promotional effect on cell replication (Yand et al., 1980; Re et al., 1992, 1996), whereas in others, stimulation of these receptors and the consequent rise in intracellular cAMP levels inhibit mitosis (Chen et al., 1998; Mitra and Carraway, 1999). β -Adrenoceptors on cancer cells thus recapitulate both the promotional and inhibitory roles of these receptors in cell replication seen in the development of normal cells (Claycomb, 1976; Slotkin et al., 1987, 1988a,b; Duncan et al., 1990). Accordingly, it might be feasible to use β -adrenoceptor agonists or antagonists as pharmacologic interventions to control the replication of cancer cells. Indeed, short-term isoproterenol treatment of C3 prostate cancer cells inhibits DNA synthesis through β -receptor-mediated increases in cAMP (Mitra and Carraway, 1999), and direct administration of membrane permeable cAMP analogs inhibits tumorigenesis of MCF-7 breast cancer cells (Chen et al., 1998).

Receptor downregulation and desensitization are major problems limiting the potential use of β -receptor agonists to control cell replication. Ordinarily, prolonged receptor stimulation uncouples receptors from response elements (desensitization) and leads to internalization and sequestration of receptor proteins (downregulation), limiting the intensity and duration of cell stimulation (Stiles, 1989). During normal development, however, we have found that these processes are poorly developed so that responses are maintained or enhanced with agonist treatment (Giannuzzi et al., 1995; Zeiders et al., 1997, 1999). This raises the possibility that loss of response may not occur

in cancer cells as well. In the current study, we evaluate that hypothesis using MDA-MB-231 cells, a human breast cancer line that expresses high levels of β -adrenoceptors (Vandewalle et al., 1990). We report that prolonged β -agonist administration maintains inhibition of DNA synthesis and suppresses cell replication even when only a small proportion of the receptors remain, so that desensitization and downregulation do not limit the effect. We also show that effects are augmented by glucocorticoids, just as is true for normal cells during development (Slotkin et al., 1994a), and also by inhibition of cAMP breakdown by theophylline.

Methods

MDA-MB-231 cells (Duke University Comprehensive Cancer Center, Durham, NC) were seeded at a density of 10^6 cells per 100 mm diameter dish and maintained in modified Minimum Essential Medium containing Earle's salts, 5% fetal bovine serum, 2 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin and 5 μ g/ml of insulin (all from Gibco, Grand Island, NY). Cells were incubated with 7.5% CO₂ at 37° C and the medium was changed every 24h. Cells were examined at 100 \times magnification for counting and morphological features. Each experiment was repeated several times with separate batches of cells. Except as otherwise indicated, all drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

DNA synthesis and content. To initiate the measurement of DNA synthesis, the medium was changed to include 1 μ Ci/ml of [³H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, MA). Incubations were carried out for 1h in the presence or absence of the appropriate drugs. At the end of that period, the medium was aspirated and cells were harvested in 3.5 ml of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid and sedimented at 1000 \times g for 15 min to precipitate macromolecules and the resultant pellet was washed once with additional trichloroacetic acid and with 75% ethanol. The final pellet was then hydrolyzed with 1 M KOH overnight at 37° C, neutralized with HCl and the DNA was then precipitated with ice-cold 5% trichloroacetic acid and sedimented at 1000 \times g for 15 min. The pellet from this final step was hydrolyzed in 5% trichloroacetic acid for 15 min at 90° C, resedimented, and an aliquot of the supernatant solution counted for [³H]thymidine incorporation. Another aliquot was assayed for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of DNA by these techniques (Bell et al., 1986). Incorporation values were corrected to the amount of DNA present in each culture to provide an index of DNA synthesis per cell.

β -Adrenoceptor binding. The medium was removed and cells were washed once with ice-cold, calcium- and magnesium-free Earle's balanced salt solution. Fresh solution was added and the cells were scraped off the dish and sedimented at 40,000 \times g for 15 min. The pellet was resuspended (Polytron, Brinkmann Instruments, Westbury, NY) in 10 mM MgCl₂, and 50 mM Tris (pH 7.4) and the homogenate was sedimented at 40,000 \times g for 15 min. The pellets were dispersed with a homogenizer (smooth glass fitted with a Teflon pestle) in the same buffer.

Each assay contained membrane suspension corresponding to \approx 5 μ g of protein and 67 pM [¹²⁵I]iodopindolol (specific activity 2200 Ci/mmol, New England Nuclear) in a final volume of 250 μ l of 145 mM NaCl, 2 mM MgCl₂, 20 mM Tris (pH 7.5) and 1 mM ascorbate. Nonspecific binding was evaluated with identical samples containing 100 μ M isoproterenol, and was typically 15% of the total binding. In some experiments, displacement of ligand binding was carried out with the specific β_1 -receptor antagonist, CGP20712A (Research Biochemicals International, Natick, MA) to identify the receptor subtype present on MDA-MB-231 cells. Scatchard determinations to identify changes in receptor number (B_{max}) or affinity (K_d) were carried out over a range of [¹²⁵I]iodopindolol concentrations from 0.02 to 1 nM.

Adenylyl cyclase activity. Cell membranes were prepared by the same procedure as for β -receptor binding, except that the buffer consisted of 250 mM sucrose, 1 mM EGTA, 10 mM Tris (pH 7.4). Aliquots of membrane preparation containing $\approx 20 \mu\text{g}$ protein were then incubated for 30 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5'-triphosphate, 10 mM MgCl_2 , 1 mg bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU phosphocreatine kinase, and $10 \mu\text{M}$ GTP in a total volume of $250 \mu\text{l}$. The enzymatic reaction was stopped by placing the samples in a $90\text{--}100^\circ\text{C}$ water bath for 5 min, followed by sedimentation at $3000 \times g$ for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits (Amersham Corp., Chicago, IL). Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay time period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, the addition of higher concentrations of GTP produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the adenylyl cyclase catalytic unit was evaluated with the response to 10 mM MnCl_2 (Chaudhry and Granneman, 1991).

The contributions of G-protein-linked processes to adenylyl cyclase were evaluated in two ways. First, to determine the net G-protein-linked response of adenylyl cyclase activity with maximal activation of all G-proteins, samples were prepared containing 10 mM NaF in the presence of GTP (Chaudhry and Granneman, 1991). Second, β -adrenoceptor-targeted effects mediated through the G-proteins were evaluated with $100 \mu\text{M}$ isoproterenol in the presence of GTP. The concentrations of all the agents used here have been found previously to be optimal for effects on adenylyl cyclase and were confirmed in preliminary experiments (Chaudhry and Granneman, 1991; Navarro et al., 1991).

Data analysis. Data are presented as means and standard errors. For each study, treatment-related differences were first evaluated by a global ANOVA, incorporating all variables in a single test. For studies of adenylyl cyclase activity, multiple measurements were made from the same membrane preparation since several different stimulants compared; in that case, stimulant was considered a repeated measure. For studies of blockade of one drug by another, or of additive or synergistic effects, the combined effects were evaluated by two-factor ANOVA with the working hypothesis dependent upon a significant interaction between the two treatments. Where significant treatment effects were identified with the global test, individual differences between treatment groups were established with Fisher's Protected Least Significant Difference.

Scatchard plots were fitted by linear regression analysis and treatment-related differences were first compared by ANCOVA. Differences in maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d , the reciprocal of receptor affinity) were then evaluated using Fisher's Protected Least Significant Difference.

Significance for main treatment effects was assumed at $p < 0.05$ and interaction terms were considered significant at $p < 0.1$ (Snedecor and Cochran, 1967). For convenience, some data are presented as a percentage of control values but statistical significance was always assessed on the unmanipulated data. Where multiple time points are presented in the same graph, the control groups are given as a single value (100%), but statistical comparisons were conducted only with the time-matched group appropriate to each treatment.

Results

MDA-MB-231 cells were in log-phase growth from 1 to 4 days after plating (Fig. 1). Over this span, DNA synthesis was maintained at a nearly constant rate and the number of cells, indicated by total DNA content, rose substantially. The increase in confluence between 1 and 4 days (more than

double) was larger than the increase in DNA content (65%), indicating that cell enlargement was also occurring over this span. Drug treatments were initiated after one day in culture and were terminated at various times during log-phase growth, no later than four days in culture.

Addition of as little as 1 nM of isoproterenol to the medium produced immediate and robust inhibition of DNA synthesis (Fig. 2). The effect was maximal at 100 nM isoproterenol and was maintained throughout a 48h exposure. At the end of that period, isoproterenol-treated cells showed a significant reduction in the number of cells, assessed by DNA content. To demonstrate that the effects of isoproterenol were mediated through β -adrenoceptors stimulating the production of cAMP, a comparison was made with the membrane permeable cAMP analog, 8-Br-cAMP, and with the effects of the β -receptor antagonist, propranolol (Fig. 3). Isoproterenol and 8-Br-cAMP were equally effective toward DNA synthesis, and the effect of isoproterenol was completely blocked by propranolol. Propranolol by itself had no effect.

In developing tissues, glucocorticoid administration can sensitize cells to β -adrenoceptor agonists by inducing receptor formation and by enhancing signaling components of the adenylyl cyclase cascade (Slotkin et al., 1994a). Accordingly, we examined whether dexamethasone enhances the ability of isoproterenol to inhibit DNA synthesis and to reduce the number of cells (Fig. 4). By itself, a 24h or 48h pretreatment with dexamethasone caused 20% inhibition of DNA synthesis and a significant reduction in cell number. When cells were pretreated for 48h with dexamethasone and then received a 2h challenge with isoproterenol, the inhibitory effects on DNA synthesis were less than additive: the net effect on DNA synthesis was no greater than that of isoproterenol alone, and the effect on DNA content was not distinguishable from that seen with just the dexamethasone pretreatment. However, when both treatments were combined for 48h, the net effects on DNA synthesis and DNA content were augmented: decrements were greater than those achieved by either treatment alone.

In addition to measurements of DNA content, drug effects on the number of MDA-MB-231 cells were established by actual cell counts (Fig. 5). Sustained isoproterenol treatment reduced the total number of cells by over 20% and a comparable effect was seen for dexamethasone. Combined treatment with dexamethasone and isoproterenol had a comparably greater effect (30%), albeit not equivalent to the summation of the two individual effects. In order to maintain cAMP levels at the highest possible value, we also treated the cells with the phosphodiesterase inhibitor, theophylline, with or without isoproterenol (Fig. 5). Theophylline completely arrested mitosis, so that addition of isoproterenol had no further effect. The selectivity of the treatments towards mitosis were confirmed by examining cell morphology (Fig. 6). Isoproterenol and dexamethasone, alone or in combination, reduced the number of cells. Theophylline caused massive reductions in cell number but the remaining cells were correspondingly larger than in the control group, indicating that the treatment did not prevent postmitotic cell growth.

To determine whether the effects of isoproterenol are shared by all cancer cells expressing β -adrenoceptors, we compared the effects on MDA-MB-231 cells with those of rat C6 glioma cells (Fig. 7). In contrast to the human breast cancer cells, C6 cells showed neither inhibition of DNA synthesis nor a reduction in DNA content over comparable periods.

The maintenance of isoproterenol-induced inhibition of DNA synthesis in MDA-MB-231 cells over a 48h span of continuous treatment suggested that either agonist-induced receptor downregulation or desensitization were not present in these cells, or alternatively, that stimulation of only a small number of receptors was sufficient to inhibit mitosis. Receptor downregulation can be selective for different subtypes and accordingly, we first evaluated which subtype was present in MDA-MB-231 cells. Using the β_1 -selective antagonist, CGP20712A, we found that displacement of [125 I]iodopindolol involved a single class of sites displaying an IC₅₀ in the μ M range (Fig. 8). For contrast, we prepared cardiac cell membranes from one day old rats (Slotkin et al., 1994a), who display predominance of the β_1 -subtype (Slotkin et al., 1994b): in this preparation, CGP20712A

displayed two IC₅₀ values, one in the nM range corresponding to the major cardiac receptor population, and a minor component which, like the MDA-MB-231 cells, displayed an IC₅₀ in the μ M range. Accordingly, the subtype expressed by MDA-MB-231 cells is almost exclusively β_2 .

We next determined whether isoproterenol treatment of MDA-MB-231 cells causes β_2 -receptor downregulation and/or uncoupling of the receptors from their ability to stimulate adenylyl cyclase. In untreated cells, adenylyl cyclase activity declined over 50% during the span of log-phase replication (Fig. 9). However, the adenylyl cyclase response to isoproterenol fell by a significantly smaller proportion than did any of the other measures and the concentration of β -receptors was maintained at the same level throughout replication and growth. Relative to total cyclase catalytic activity (Mn^{2+}), the isoproterenol response actually increased over the course of culturing. After 1 day in culture, isoproterenol evoked $75 \pm 3\%$ of the total catalytic response exemplified by Mn^{2+} , whereas after 3-4 days in culture, the two stimulations were indistinguishable: isoproterenol evoked $97 \pm 3\%$ of the total response ($p < 0.0001$ compared to the proportion after 1 day in culture).

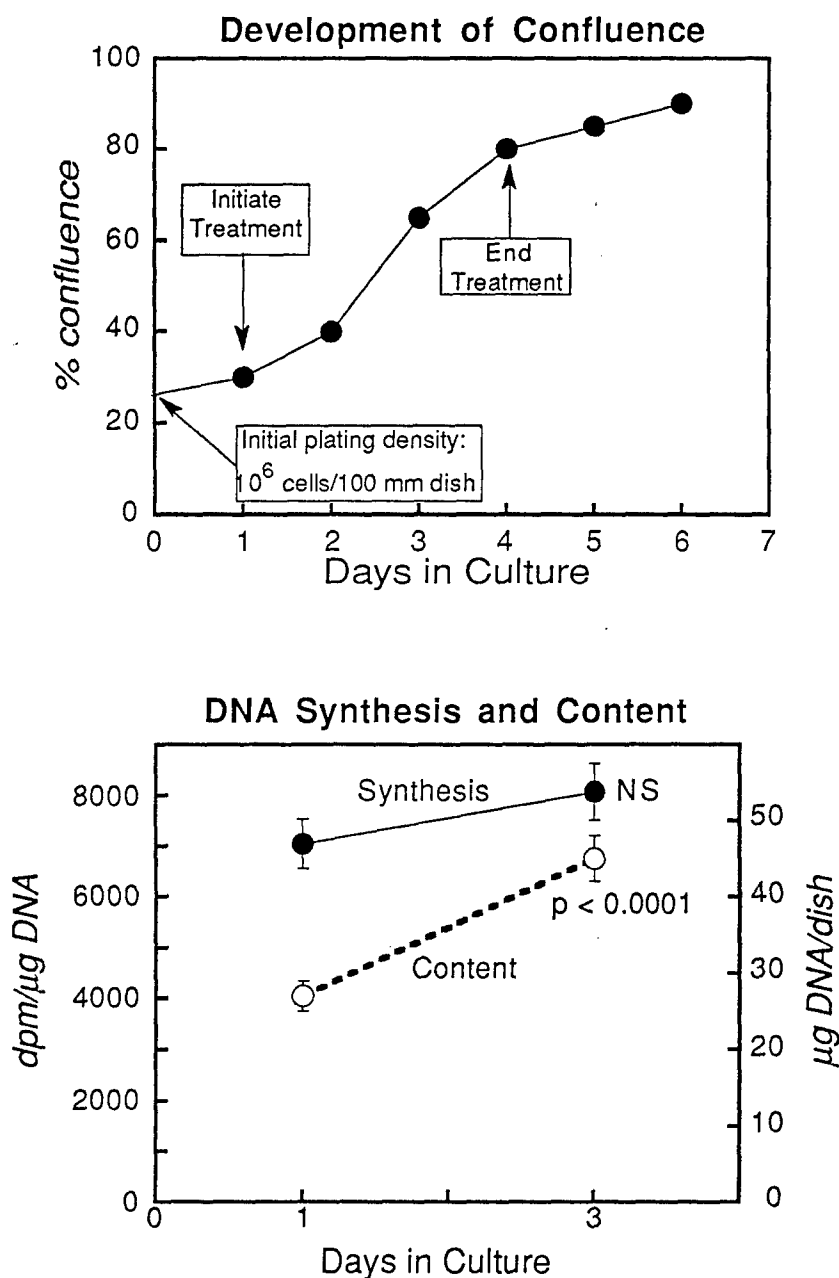
Despite the fact that isoproterenol-induced inhibition of DNA synthesis was maintained throughout a 48h drug exposure, receptor downregulation was apparent immediately upon introduction of the drug (Fig. 10). A concentration-dependent reduction in receptor binding was evident within 1h, with nearly complete downregulation by 24h. Receptor binding then remained at 5-10% of control values throughout 72h of exposure. Isoproterenol concentrations as low as 1 nM produced significant, albeit submaximal, reductions in receptor binding after 24h of exposure. Scatchard analysis confirmed that the loss of receptor binding reflected a decrease in the number of receptors as measured by maximal binding, rather than a change in receptor affinity as monitored by the K_d . In additional studies, we found that a 2h daily isoproterenol exposure was sufficient to cause full receptor downregulation. We treated cells for three days in succession, using 100 μ M isoproterenol for 2h each day, followed in each case by 22h without drug; 22h after the third day's exposure, receptor binding was only $6 \pm 1\%$ of control values ($n=6$, $p < 0.0001$). Similarly, even when we reduced the concentration to 1 μ M with exposure for 2h per day over a two day span, receptor measurements made 22h after the last exposure still indicated robust downregulation ($8 \pm 1\%$ of control, $n=6$, $p < 0.0001$).

We also determined whether agonist-induced receptor downregulation was accompanied by loss of the adenylyl cyclase response to isoproterenol (Fig. 11). Treatment of cells with 1 μ M isoproterenol for 2h, which caused approximately a 25% reduction in β -receptor binding, also elicited a comparable loss of the membrane response of adenylyl cyclase to isoproterenol. However, changes at the level of G-protein function were evident: basal enzyme activity, measured in the presence of GTP, also showed significant and immediate reductions, and the response to maximal G-protein activation by fluoride was impaired by a small amount. After a 48h exposure to isoproterenol, desensitization of the membrane adenylyl cyclase response to isoproterenol reached 75%, not quite as large as the degree of receptor downregulation; again, effects on basal adenylyl cyclase activity also were present, but were not as notable as the change in the β -receptor-mediated response. At no point did we note any decline in the expression or catalytic activity of adenylyl cyclase itself, as monitored by the effect of Mn^{2+} .

In light of the enhanced effect on cell replication of the combination of dexamethasone and isoproterenol treatment, we also examined their interaction at the levels of β -adrenoceptor binding and adenylyl cyclase activity. Pretreatment of cells with dexamethasone for 24h had no effect on β -receptor binding (Fig. 12, top panel). However, when the pretreatment was combined with a subsequent, 4h exposure to isoproterenol, it enhanced the downregulation caused by the receptor agonist. Dexamethasone had only small effects on adenylyl cyclase activity (Fig. 12, bottom panel). By itself, dexamethasone lowered the membrane response to isoproterenol by a few percent. When dexamethasone pretreatment was superimposed on short-term isoproterenol treatment of the cells, basal adenylyl cyclase activity was inhibited slightly less than with

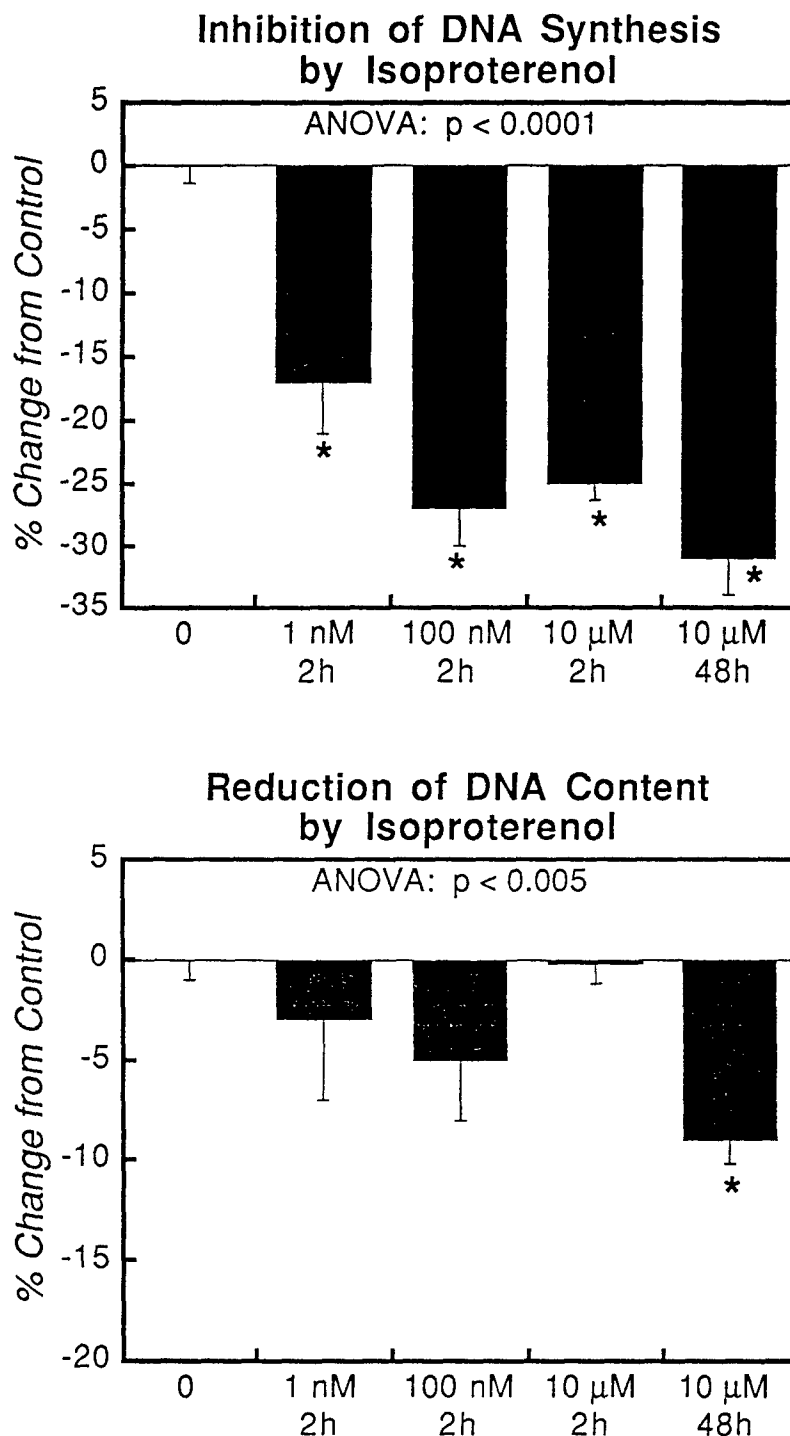
isoproterenol alone and the fluoride response was inhibited somewhat more. However, the agonist-induced desensitization of the specific response to isoproterenol was just as prominent after dexamethasone pretreatment as it was without pretreatment.

Fig. 1



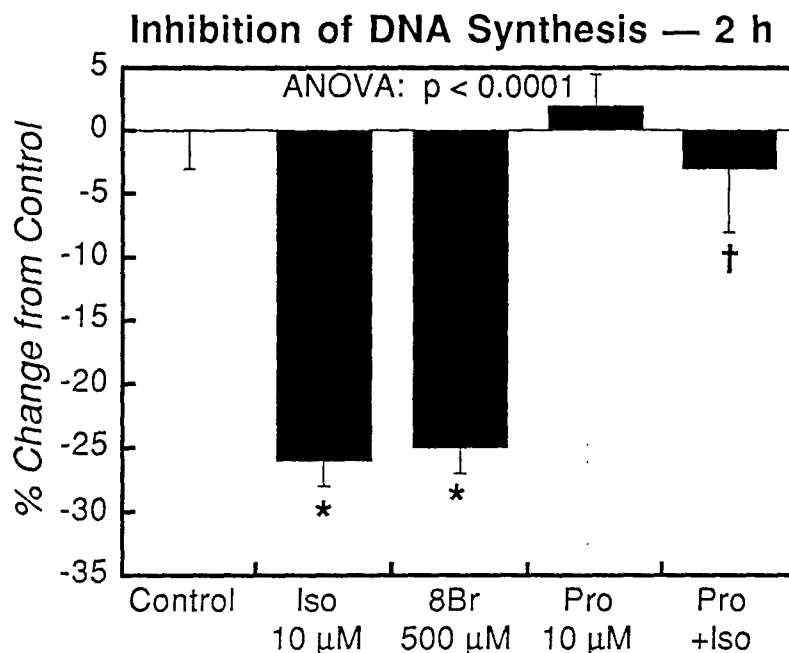
Cell replication and growth characteristics of MDA-MB-231 cells in culture. Data represent means and standard errors obtained from 40-60 determinations at each time point. For all experiments, treatments were initiated after one day in culture and were terminated no later than four days in culture.

Fig. 2



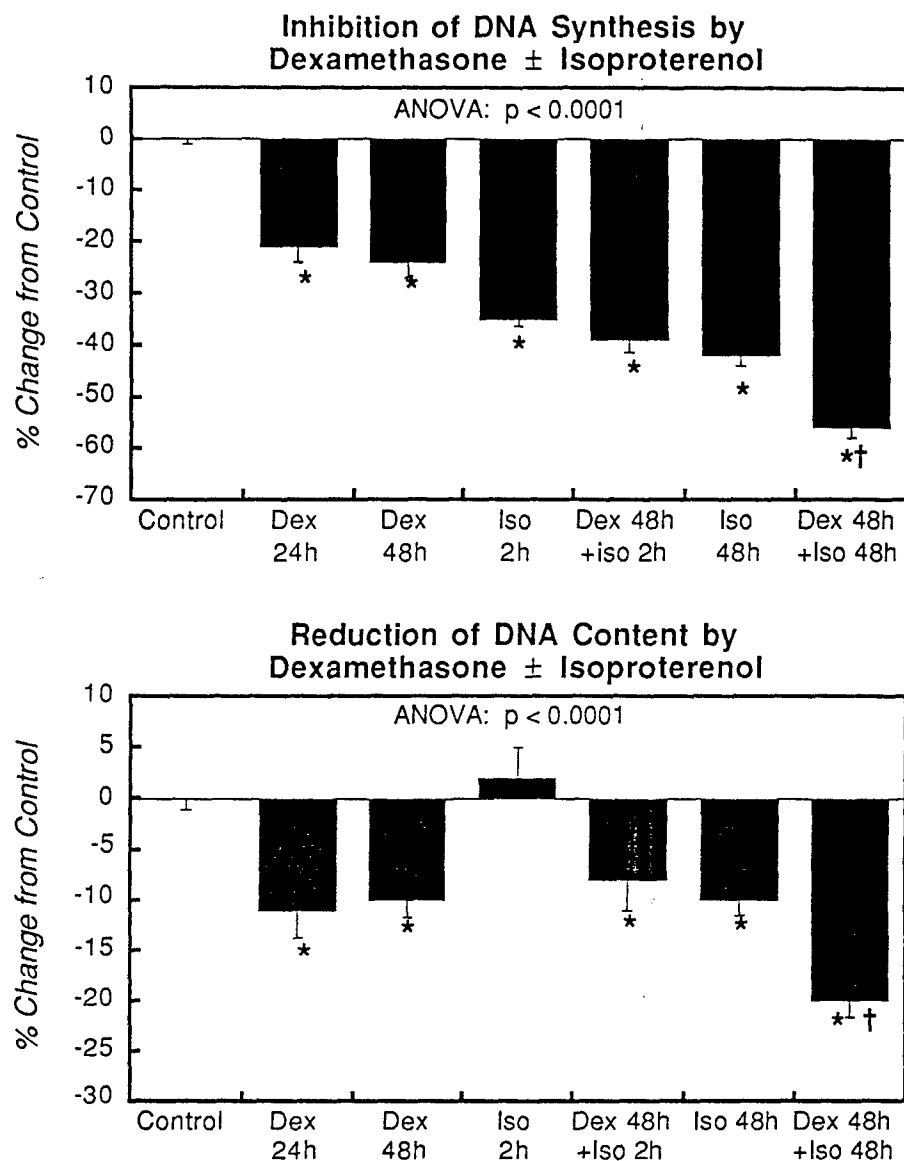
Effects of isoproterenol treatment on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 12-96 determinations for each concentration and time point. Isoproterenol was added for 2h or 48h, with inclusion of [^3H]thymidine for the final hour. ANOVA across all treatments appears at the top of each panel and asterisks denote individual treatments that differ significantly from the control.

Fig. 3



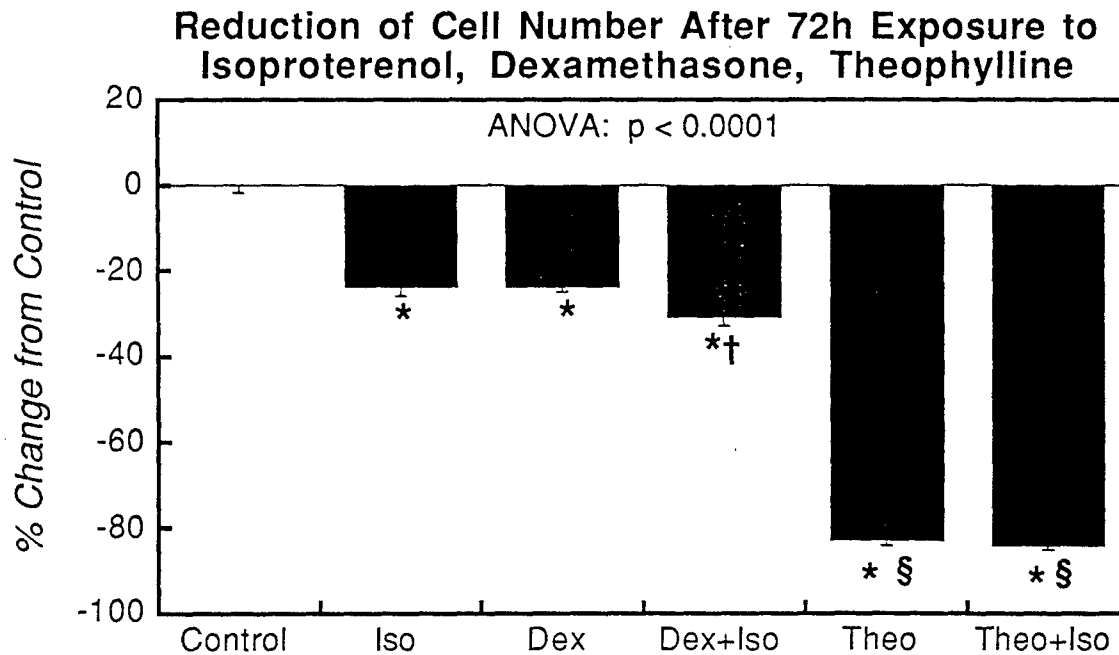
Effects of a 2h treatment with isoproterenol (Iso), 8-bromo-cAMP (8Br), or propranolol (Pro) treatment on DNA synthesis, presented as the percentage change from control values. Data represent means and standard errors obtained from 10-26 determinations for each concentration and time point. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the dagger denotes a significant difference between Pro + Iso and Iso alone. In addition, two-factor ANOVA for the effects of propranolol on the isoproterenol response indicated complete blockade ($p < 0.002$ for the main effect of isoproterenol, $p < 0.02$ for the main effect of propranolol, $p < 0.05$ for the interaction of the two treatments). None of the treatments produced a significant change in DNA content (data not shown).

Fig. 4

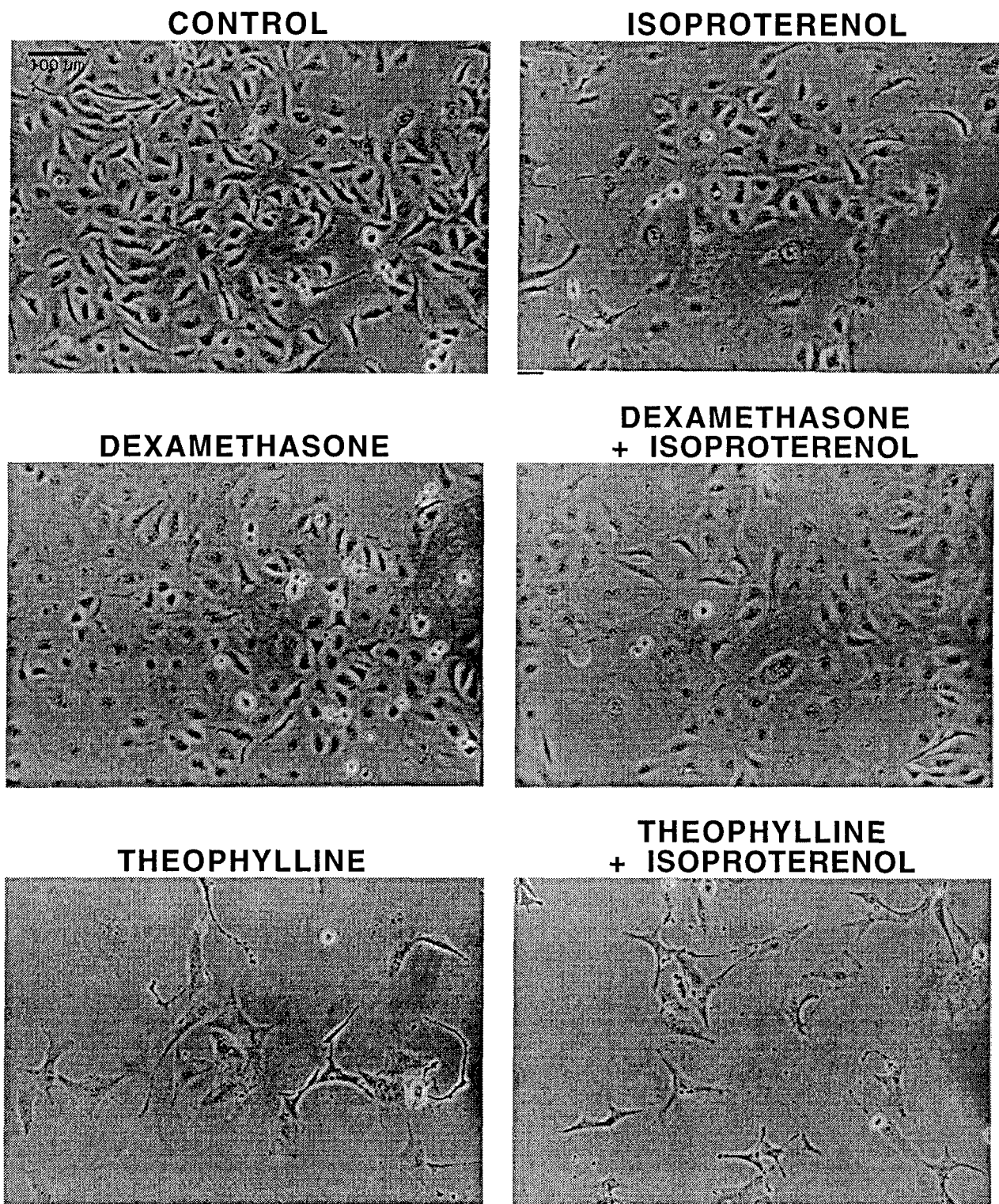


Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 11-54 determinations for each treatment. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the daggers denote significant differences between Dex 48h + Iso 48h, and the corresponding treatments with Dex or Iso alone. In addition, for DNA synthesis, two-factor ANOVA (dexamethasone 48h \times isoproterenol 2h) indicates significant main effects of dexamethasone ($p < 0.0005$) and isoproterenol ($p < 0.0001$) but no interaction between the two treatments; with 48h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each) as well as a significant interaction of dexamethasone \times isoproterenol ($p < 0.002$). For DNA content, two-factor ANOVA (dexamethasone 48h \times isoproterenol 2h) indicates a significant main effect of dexamethasone ($p < 0.009$) but no effect of isoproterenol; with 48h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each).

Fig. 5

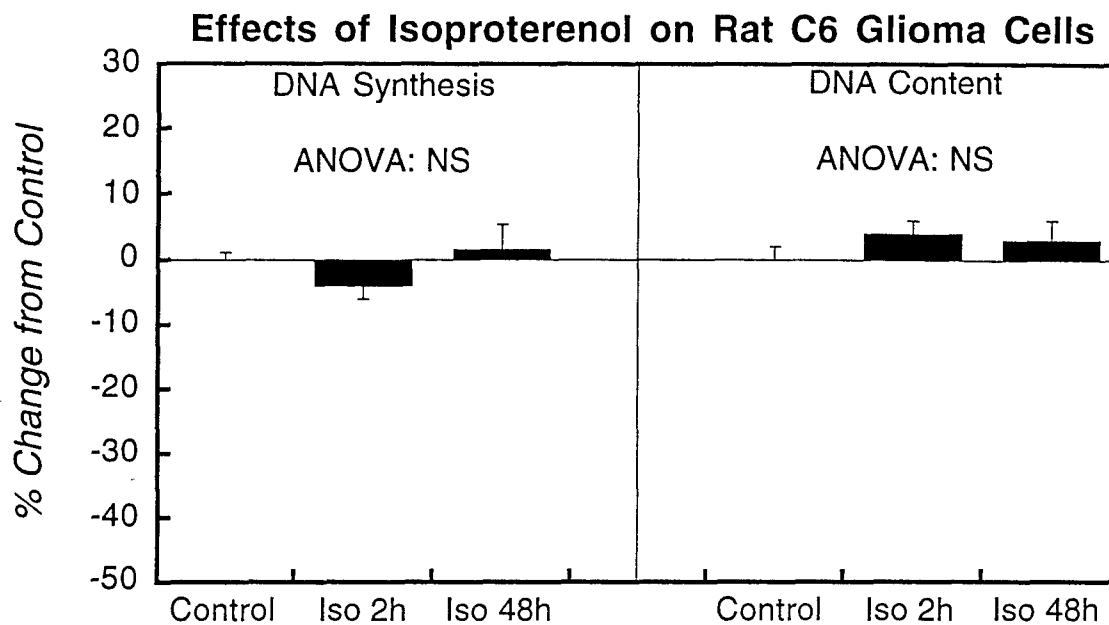


Effects of a 72h treatment with isoproterenol (Iso), dexamethasone (Dex), or theophylline (Theo) on cell number, presented as the percentage change from control values. Data represent means and standard errors obtained from 10-22 determinations for each treatment. ANOVA across all treatments appears at the top of each panel. Asterisks denote individual treatments that differ significantly from the control; the dagger denotes a significant difference between Dex + Iso and either treatment alone; § denotes significant differences between theophylline with or without isoproterenol, as compared to all other treatments. In addition, two-factor ANOVA across the dexamethasone and isoproterenol treatments indicates significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of dexamethasone \times isoproterenol ($p < 0.03$). Across the theophylline and isoproterenol groups, there were significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of theophylline \times isoproterenol ($p < 0.0001$).

Fig. 6

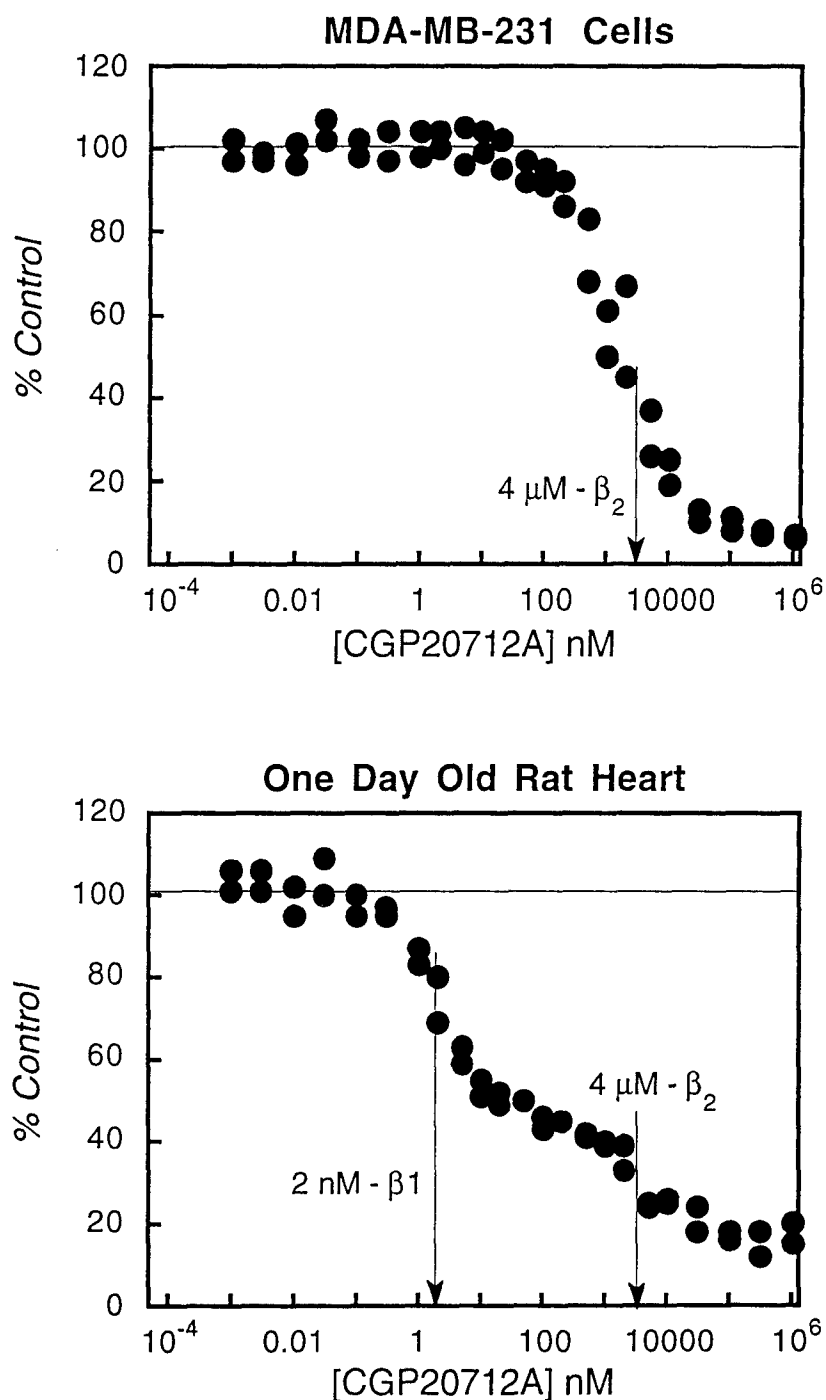
Phase-contrast microscopic appearance of MDA-MB-231 cells after a 72h treatment with isoproterenol, dexamethasone, or theophylline. Scale bar appears in the upper left panel.

Fig. 7



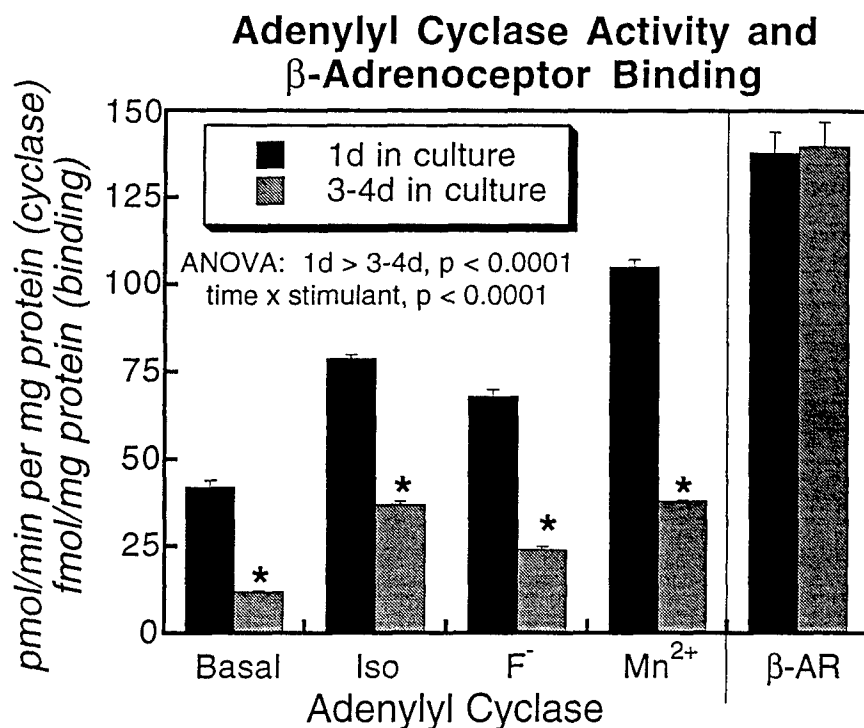
Effects of 10 μ M isoproterenol (Iso) on DNA synthesis and content in rat C6 glioma cells, presented as the percentage change from control values. Data represent means and standard errors obtained from 11-30 determinations for each time point. ANOVA across all treatments appears within each panel.

Fig. 8



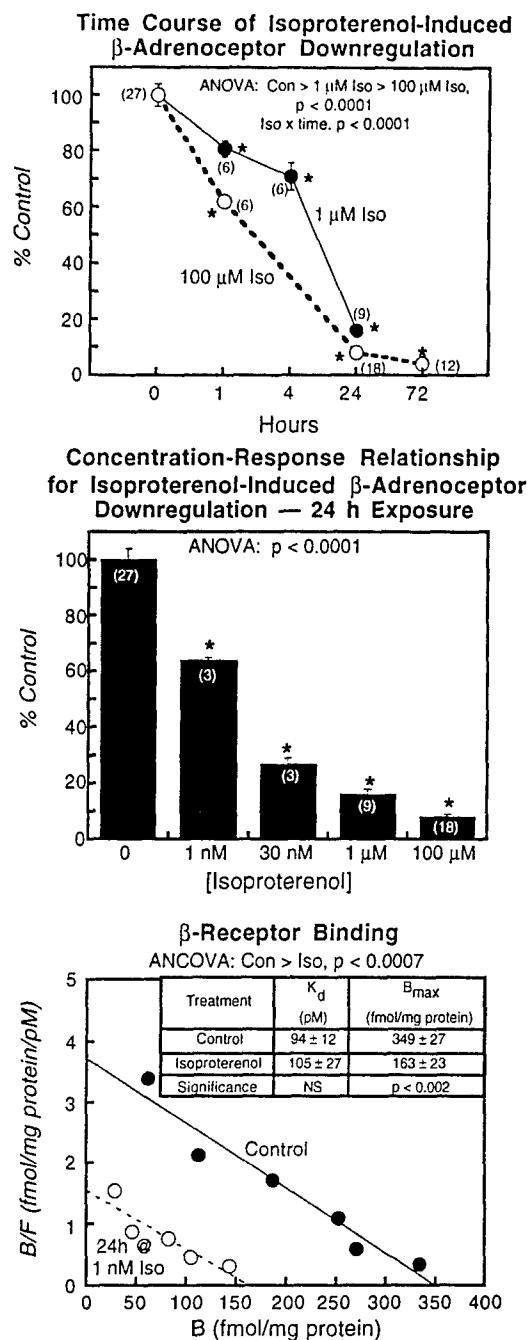
Binding of [125 I]iodopindolol to β -adrenoceptors in cell membranes prepared from MDA-MB-231 cells and from one day old rat heart, and its displacement by the β_1 -specific antagonist, CGP20712A. Each data point represents an individual determination, shown as the percentage of values obtained in the absence of displacer. The [125 I]iodopindolol concentration was 67 pM.

Fig. 9



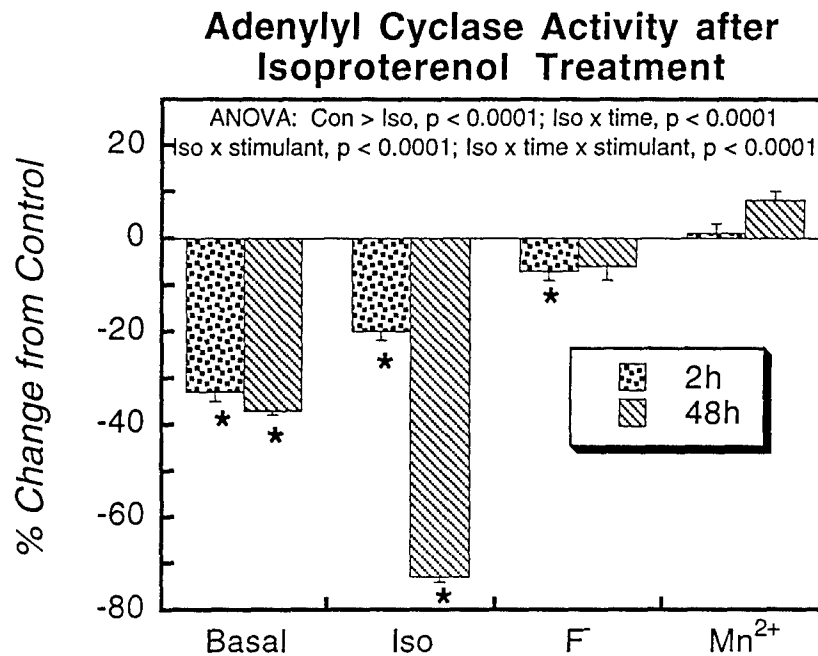
Adenylyl cyclase activity and β-adrenoceptor binding in MDA-MB-231 cells during cell replication and growth in culture. Cells were cultured for the indicated time periods and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μM isoproterenol (Iso), 10 mM fluoride (F⁻) or 10 mM Mn²⁺. Data represent means and standard errors obtained from 6-24 determinations for each measurement at each time point. ANOVA across both time points and all stimulants appears within the panel and asterisks denote measures for which the values after 3-4 days in culture are significantly lower than the initial values. In addition, the cyclase response to isoproterenol declines less than the response to other stimulants ($p < 0.0001$).

Fig. 10



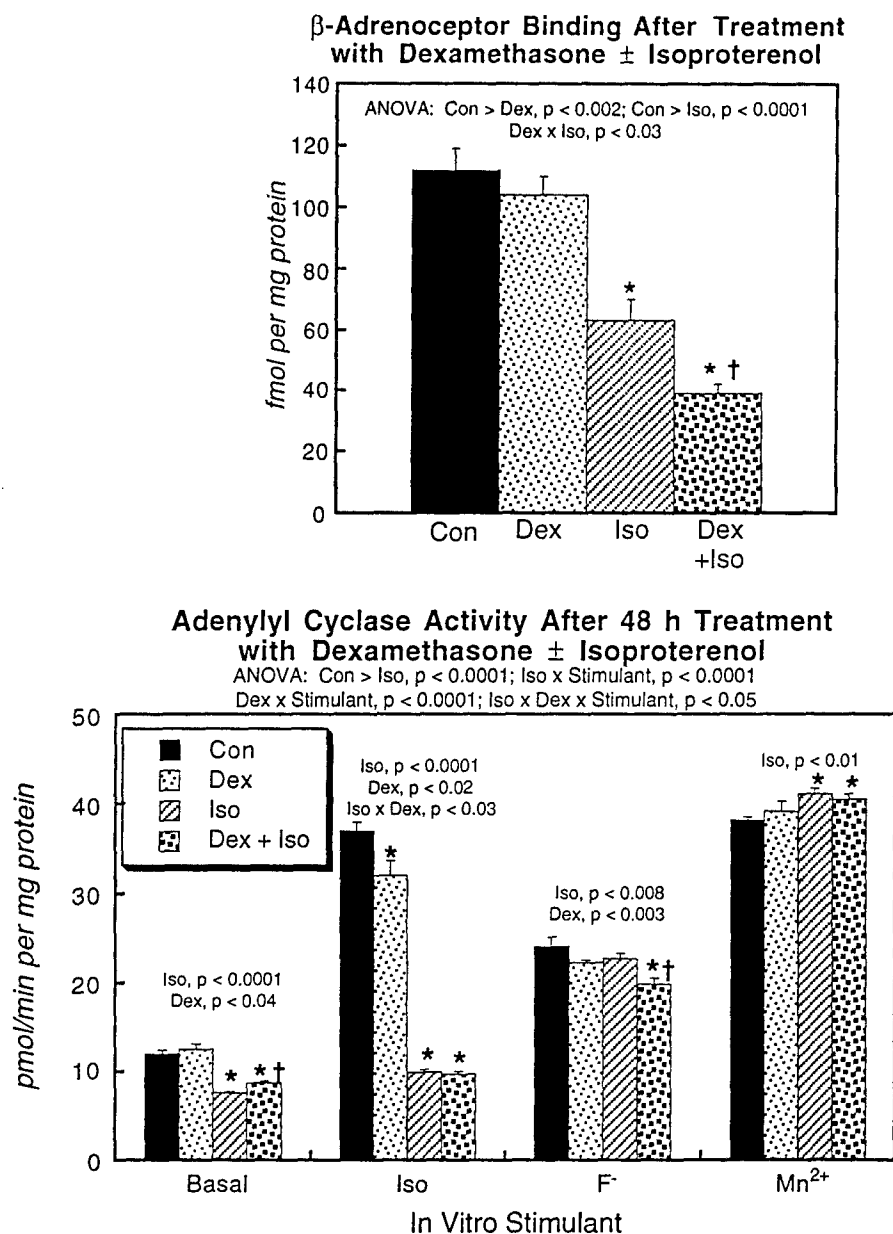
β -Adrenoceptor downregulation caused by isoproterenol treatment. Data represent means and standard errors obtained from the number of determinations shown in parentheses, determined as the percentage change from control values. In the top and middle panels, ANOVA across all time points or treatments appears at the top and asterisks denote individual values that differ significantly from the control. In the bottom panel, ANCOVA appears for the overall differences between Scatchard plots in control and isoproterenol-treated (Iso) cells.

Fig. 11



Desensitization of adenylyl cyclase evoked by exposure to 1 μ M isoproterenol. Cells were treated with isoproterenol for the indicated time period and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μ M isoproterenol (Iso), 10 mM fluoride (F⁻) or 10 mM Mn²⁺. Data represent means and standard errors obtained from 6-12 determinations at each time point, determined as the percentage change from control values. ANOVA across all stimulants and both time points appears at the top and asterisks denote individual values that differ significantly from the control. Two-factor ANOVAs (treatment \times time) were also assessed for each variable. Across both time points, basal activity was significantly reduced ($p < 0.0001$ for the main effect of isoproterenol), the response to isoproterenol was reduced in a time-dependent fashion ($p < 0.0001$ for the main effect, $p < 0.0001$ for the treatment \times time interaction), the response to fluoride was reduced ($p < 0.0001$ for the main effect), and the response to Mn²⁺ was unaffected.

Fig. 12



Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on β -adrenoceptor binding (top) and adenylyl cyclase activity (bottom). Cells were pretreated with 10 μ M dexamethasone for 24h (top) or 48h (bottom) and were then exposed to 1 μ M isoproterenol for 4h. For adenylyl cyclase, enzyme activity was determined under basal conditions or in the presence of 100 μ M isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+} . Data represent means and standard errors obtained from 6-12 determinations for each treatment. For receptor binding, ANOVA across all treatments appears at the top; for adenylyl cyclase, ANOVA across all treatments and *in vitro* stimulants appears at the top, and lower order ANOVAs are shown for *in vitro* stimulant with each cluster of bars. Asterisks denote individual treatments that differ significantly from the control and the daggers denotes significant difference of combined treatment from the effects of dexamethasone or isoproterenol alone.

7. KEY RESEARCH ACCOMPLISHMENTS

- β -Adrenoceptors (β AR) are present on the cell surface in a number of different human breast cancer cell lines, including those that are nonresponsive to estrogen, and that are thus resistant to antiestrogen therapy.
- Exposure of MDA MB-231 human breast cancer cells to isoproterenol, a β AR agonist, caused an immediate reduction in DNA synthesis. The effect was mimicked by administration of membrane permeable cyclic AMP analogs, and showed additive actions with glucocorticoids.
- With continued exposure, the effect of isoproterenol was maintained, despite downregulation of β AR and uncoupling of receptors from adenylyl cyclase; apparently, only a small proportion of β AR need to be stimulated to evoke mitotic inhibition.
- After 48-72 h of isoproterenol treatment, a significant reduction in cell number was obtained. Addition of a glucocorticoid, dexamethasone, enhanced the loss of cells. With addition of theophylline, which inhibits the breakdown of cyclic AMP, mitotic arrest was nearly complete.
- β AR-targeted therapy may offer a chance to slow the growth and spread of some estrogen nonresponsive breast cancers. Since the effects on the cancer cells do not desensitize, whereas effects on normal cells do, effective regimens may be designed that minimize side effects. Attacking a cell surface receptor should remain an effective approach even after the emergence of multidrug resistance, which reduces the effectiveness only of drugs targeting intracellular sites.

8. REPORTABLE OUTCOMES

Manuscript: Slotkin, T.A., J. Zhang, R. Dancel, S.J. Garcia, C. Willis and F.J. Seidler, β -Adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells, *Journal of Pharmacology and Experimental Therapeutics*, submitted for publication.

9. CONCLUSIONS

Among the epithelial, endocrine and secretory cancer cell lines that express β -adrenoceptors, MDA-MB-231 human breast cancer cells exhibit comparatively high concentrations (Vandewalle et al., 1990; Draoui et al., 1991; Marchetti et al., 1991; Re et al., 1992, 1996). The B_{\max} found here, 350 fmol/mg protein, exceeds the receptor concentration found in typical noradrenergic target tissues by over an order of magnitude (Thai et al., 1996) and is similar to the level found in brain regions enriched in noradrenergic projections. It is thus of critical interest that stimulation of these receptors leads to immediate inhibition of DNA synthesis and, with prolonged exposure, reductions in the total number of cancer cells. This effect was not shared by another cancer cell line, C6 rat glioma cells, that express a different β -receptor subtype at lower levels: whereas we found that MDA-MB-231 cells express β_2 -receptors exclusively, C6 cells express primarily the β_1 -subtype (Homburger et al., 1981).

Isoproterenol-induced inhibition of DNA synthesis in the breast cancer cell line exhibited the characteristic properties of β -receptor actions mediated through adenylyl cyclase: complete blockade by isoproterenol, and sharing of the effect by a membrane-permeable cAMP analog. What was unexpected, however, was the fact that isoproterenol's effect did not disappear with prolonged treatment, and in fact, was maintained at exactly the same level as the initial effect. Ordinarily, β -adrenoceptor agonists elicit downregulation and desensitization over a time frame of a few minutes to hours (Stiles, 1989), and therefore a loss of effect was expected here. The first hint of atypical regulation of β -receptor signaling was provided simply by monitoring adenylyl

cyclase responses under control conditions. Over a course of several days in culture, adenylyl cyclase activity declined, accompanied by a loss of responsiveness to stimulants acting either at the level of β -receptors (isoproterenol), G-proteins (fluoride), or cyclase itself (Mn^{2+}). However, the enzymatic response to isoproterenol showed a smaller decline than for any other stimulant, that is, the β -adrenoceptor response actually increased relative to the total amount of catalytic activity. In fact, after 3-4 days in culture, isoproterenol elicited the maximal possible activation of adenylyl cyclase, since the activity was indistinguishable from that seen with addition of Mn^{2+} . In addition, the response to isoproterenol, which selectively activates G_s , was significantly greater than that to fluoride, which causes activation of both stimulatory and inhibitory G-proteins. The profound response to isoproterenol is unusual, since in most tissues, isoproterenol is incapable of eliciting adenylyl cyclase activation equaling that of direct G-protein or cyclase stimulants (Navarro et al., 1991; Giannuzzi et al., 1995; Wagner et al., 1995; Zeiders et al., 1997). The number of β -receptors also kept pace with cell division and growth, so that the overall concentration of receptors remained unchanged throughout 4 days in culture, a period in which the number and size of cells increased substantially.

Given the maintenance of the ability of isoproterenol to inhibit DNA synthesis, we expected to see failure of receptor downregulation and/or desensitization, paralleling the situation during differentiation of normal cells possessing these receptors (Navarro et al., 1991; Giannuzzi et al., 1995; Zeiders et al., 1997). Surprisingly, isoproterenol caused immediate and robust receptor downregulation, accompanied by a parallel loss of the ability of receptor activation to stimulate adenylyl cyclase activity. Downregulation reached over 90% within 24h and the adenylyl cyclase response was desensitized by 75% throughout the period in which inhibition of DNA synthesis was maintained. These results thus indicate that downregulation and desensitization do occur, but that the ability of receptor stimulation to inhibit DNA synthesis and cell acquisition requires activation of only a very small number of receptors. This raises the possibility that there may be adaptations in the signaling pathway downstream from receptors, G-proteins and cyclase, that serve to maintain the net effect of receptor stimulation. In keeping with this view, we have found that, during brain development, the initial stimulation of β -receptors "programs" cAMP response elements so as to preserve or enhance the response of gene expression to adrenergic input (Wagner et al., 1994, 1995). If similar events occur in MDA-MB-231 cells, then adaptations of downstream elements may preserve the effects on cell replication in the face of receptor downregulation and desensitization. Accordingly, a logical next step is to look at transcription factors and genes targeted by β -receptor stimulation and their role in the antimitotic effect of isoproterenol.

From both the standpoints of mechanism and therapeutics, our findings of augmented effects with cotreatment of dexamethasone or theophylline are potentially important. Initially, we expected dexamethasone to enhance the response to isoproterenol because glucocorticoids induce the formation of β_2 -receptors in normal cells (Davies and Lefkowitz, 1984), especially during cell differentiation (Slotkin et al., 1994a); furthermore, these hormones also induce G-proteins and adenylyl cyclase in the fetus (Slotkin et al., 1994a). Dexamethasone treatment by itself inhibited DNA synthesis in MDA-MB-231 cells leading eventually to a reduction in cell number. In addition, when dexamethasone was combined with isoproterenol treatment, we saw even greater inhibition of DNA synthesis and loss of cells. However, when we examined the mechanism underlying the combined effect, we found that dexamethasone was not capable of preventing agonist-induced β -receptor downregulation or desensitization. Accordingly, the results again imply that actions downstream from the generation of cAMP are recruited to maintain the antimitotic response to receptor stimulation.

The second cotreatment that we examined was the phosphodiesterase inhibitor, theophylline. This drug alone had the greatest effect on mitosis: after several days in culture, the number of cells appeared to be no greater than that originally plated. Theophylline has a greater effect than isoproterenol because it interrupts the ability of phosphodiesterase to limit the rise of intracellular

cAMP levels. However, in light of the findings for isoproterenol and dexamethasone, it is again possible that theophylline may influence gene expression downstream from cAMP generation, or alternatively, may act through other surface receptors (*e.g.* adenosine receptors) whose expression has not been explored in these cell lines.

"SO WHAT"

Regardless of the ancillary mechanisms involved in β -adrenoceptor-mediated inhibition of mitosis in MDA-MB-231 breast cancer cells, the fact that inhibition does not disappear with receptor downregulation and desensitization raises the possibility for therapeutic strategies employing receptor agonists, alone or in combination with glucocorticoids and phosphodiesterase inhibitors. The cell line studied here, for example, is estrogen-insensitive and is thus nonresponsive to standard antiestrogen therapies. Furthermore, interventions operating at the level of cell surface receptors, such as β -adrenoceptors, do not require penetration of drug to the interior of the cell, and thus would not be subject to loss of effect from induction of transporters in multidrug resistance. From a mechanistic standpoint, receptor input is "upstream" from genes, whose mutations lead to constitutive activation and cell proliferation (Mirossay et al., 1992; Moens et al., 1993; Wagner et al., 1994); receptor stimulation could thus limit the net effects of adverse mutations on cell cycle control by restricting expression of these genes. Given that desensitization effectively terminates the physiological effects of β -agonists in normal cells, therapeutic interventions based on receptor targeting should have only short-term side effects relative to the maintenance of effect in the target cell population; furthermore, the pharmacokinetics and toxicity of these agents are well-established. Neurotransmitter-based therapeutic strategies should be explored in an *in vivo* model to establish the potential utility of this general approach. If successful, screening of human cancers for the presence of β -adrenoceptors, or indeed, of other cell surface receptors, along with *in vitro* evaluation of the response of the cells to receptor agonists or antagonists, may establish new treatment strategies.

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11. APPENDICES

Attachment — Manuscript: Slotkin, T.A., J. Zhang, R. Dancel, S.J. Garcia, C. Willis and F.J. Seidler, β -Adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells, *Journal of Pharmacology and Experimental Therapeutics*, submitted for publication.

**β -Adrenoceptor Signaling and Its Control of Cell Replication
in MDA-MB-231 Human Breast Cancer Cells**

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(appendix)

Running title: β -Adrenoceptors in breast cancer

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of references: 40

Abbreviations: ANCOVA, analysis of covariance

ANOVA, analysis of variance

cAMP, cyclic adenosine-3',5'-monophosphate

ABSTRACT

MDA-MB-231 human breast cancer cells express high β -adrenoceptor levels, predominantly the β_2 subtype. Receptor stimulation by isoproterenol evoked immediate reductions in DNA synthesis which were blocked completely by propranolol and were of the same magnitude as effects elicited by high concentrations of 8-Br-cAMP. Isoproterenol-induced inhibition of DNA synthesis was maintained throughout several days of exposure, resulting in a decrement in total cell number, and the effects were augmented by cotreatment with dexamethasone; an even greater effect was seen when cAMP breakdown was inhibited by theophylline, with or without addition of isoproterenol. Despite the persistent effect of isoproterenol, receptor downregulation was evident with as little as 1h of treatment and over 90% of the receptors were lost within 24h. Receptor downregulation was paralleled by homologous desensitization of the adenylyl cyclase response to β -adrenoceptor stimulation. Despite the fact that dexamethasone augmented the effects of isoproterenol on DNA synthesis, it did not prevent receptor downregulation or desensitization. These results indicate β -adrenoceptors are effectively linked, through cAMP, to the termination of cell replication in MDA-MB-231 human breast cancer cells, and that activation of only a small number of receptors is sufficient for a maximal effect. Novel pharmacologic strategies that focus on cell surface receptors operating through adenylyl cyclase may offer opportunities to combat cancers that are unresponsive to hormonal agents, or that have developed multidrug resistance.

In addition to their role as neurotransmitters and "stress" hormones, catecholamines play a trophic role in the control of cell replication and differentiation in target cells that express adrenergic receptors. Lower organisms, such as sea urchins, overexpress norepinephrine, epinephrine and other biogenic amines during critical developmental periods in which these amines control cell replication and differentiation (Buznikov et al., 1970). In mammals, "spikes" of adrenergic activity also modulate the rate of cell replication and differentiation and thus control the architectural modeling of adrenergic target tissues (Vernadakis and Gibson, 1974; Claycomb, 1976; Lovell, 1982; Slotkin et al., 1987, 1988a). The importance of adrenergic control of mammalian cell development has recently been pointed out by the lethal effects of gene knockouts that eliminate the ability to synthesize norepinephrine or to express β -receptors; these animals die *in utero* from disruption of cardiac cell replication/differentiation and consequent dysmorphogenesis (Thomas et al., 1995; Zhou et al., 1995; Rohrer et al., 1996). The critical period for adrenergic control of these events terminates as cells exit mitosis and approach terminal differentiation, so that the sensitivity to adrenergic stimulation of cell replication disappears in adulthood except for a few tissues that undergo continual renewal (Claycomb, 1976; Slotkin et al., 1987; Wagner et al., 1994; Zeng et al., 1996).

It is thus of critical importance that, with carcinogenic redifferentiation, many cell types, including epithelial cancers and cancers of secretory cells, re-express β -adrenergic receptors (Ling et al., 1992; MacEwan and Milligan, 1996; Re et al., 1996; Canova et al., 1997; Mitra and Carraway, 1999), which can once again resume their role in the control of cell replication (Re et al., 1992, 1996; Mitra and Carraway, 1999). In some cell lines, β -adrenergic stimulation elicits a small, promotional effect on cell replication (Yand et al., 1980; Re et al., 1992, 1996), whereas in others, stimulation of these receptors and the consequent rise in intracellular cAMP levels inhibit mitosis (Chen et al., 1998; Mitra and Carraway, 1999). β -Adrenoceptors on cancer cells thus recapitulate both the promotional and inhibitory roles of these receptors in cell replication seen in the development of normal cells (Claycomb, 1976; Slotkin et al., 1987, 1988a,b; Duncan et al., 1990). Accordingly, it might be feasible to use β -adrenoceptor agonists or antagonists as

pharmacologic interventions to control the replication of cancer cells. Indeed, short-term isoproterenol treatment of C3 prostate cancer cells inhibits DNA synthesis through β -receptor-mediated increases in cAMP (Mitra and Carraway, 1999), and direct administration of membrane permeable cAMP analogs inhibits tumorigenesis of MCF-7 breast cancer cells (Chen et al., 1998).

Receptor downregulation and desensitization are major problems limiting the potential use of β -receptor agonists to control cell replication. Ordinarily, prolonged receptor stimulation uncouples receptors from response elements (desensitization) and leads to internalization and sequestration of receptor proteins (downregulation), limiting the intensity and duration of cell stimulation (Stiles, 1989). During normal development, however, we have found that these processes are poorly developed so that responses are maintained or enhanced with agonist treatment (Giannuzzi et al., 1995; Zeiders et al., 1997, 1999). This raises the possibility that loss of response may not occur in cancer cells as well. In the current study, we evaluate that hypothesis using MDA-MB-231 cells, a human breast cancer line that expresses high levels of β -adrenoceptors (Vandewalle et al., 1990). We report that prolonged β -agonist administration maintains inhibition of DNA synthesis and suppresses cell replication even when only a small proportion of the receptors remain, so that desensitization and downregulation do not limit the effect. We also show that effects are augmented by glucocorticoids, just as is true for normal cells during development (Slotkin et al., 1994a), and also by inhibition of cAMP breakdown by theophylline.

METHODS

MDA-MB-231 cells (Duke University Comprehensive Cancer Center, Durham, NC) were seeded at a density of 10^6 cells per 100 mm diameter dish and maintained in modified Minimum Essential Medium containing Earle's salts, 5% fetal bovine serum, 3 mM glutamine, 100 IU/ml of penicillin, 0.1 mg/ml of streptomycin and 5 μ g/ml of insulin (all from Gibco, Grand Island, NY). Cells were incubated with 7.5% CO₂ at 37° C and the medium was changed every 24h. Cells were examined at 100 \times magnification for counting and morphological features. Each experiment was repeated several times with separate batches of cells. Except as otherwise indicated, all drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

DNA synthesis and content. To initiate the measurement of DNA synthesis, the medium was changed to include 1 μ Ci/ml of [³H]thymidine (specific activity, 2 Ci/mmol; New England Nuclear, Boston, MA). Incubations were carried out for 1h in the presence or absence of the appropriate drugs. At the end of that period, the medium was aspirated and cells were harvested in 3.5 ml of ice-cold water. Duplicate aliquots of each sample were treated with 10% trichloroacetic acid and sedimented at 1000 \times g for 15 min to precipitate macromolecules and the resultant pellet was washed once with additional trichloroacetic acid and with 75% ethanol. The final pellet was then hydrolyzed with 1 M KOH overnight at 37° C, neutralized with HCl and the DNA was then precipitated with ice-cold 5% trichloroacetic acid and sedimented at 1000 \times g for 15 min. The pellet from this final step was hydrolyzed in 5% trichloroacetic acid for 15 min at 90° C, resedimented, and an aliquot of the supernatant solution counted for [³H]thymidine incorporation. Another aliquot was assayed for DNA spectrophotometrically by absorbance at 260 nm. Previous work has demonstrated quantitative recovery of DNA by these techniques (Bell et al., 1986). Incorporation values were corrected to the amount of DNA present in each culture to provide an index of DNA synthesis per cell.

β -Adrenoceptor binding. The medium was removed and cells were washed once with ice-cold, calcium- and magnesium-free Earle's balanced salt solution. Fresh solution was added and the cells were scraped off the dish and sedimented at 40,000 \times g for 15 min. The pellet was

resuspended (Polytron, Brinkmann Instruments, Westbury, NY) in 10 mM MgCl₂, and 50 mM Tris (pH 7.4) and the homogenate was sedimented at 40,000 × g for 15 min. The pellets were dispersed with a homogenizer (smooth glass fitted with a Teflon pestle) in the same buffer.

Each assay contained membrane suspension corresponding to ≈5 µg of protein and 67 pM [¹²⁵I]iodopindolol (specific activity 2200 Ci/mmol, New England Nuclear) in a final volume of 250 µl of 145 mM NaCl, 2 mM MgCl₂, 20 mM Tris (pH 7.5) and 1 mM ascorbate. Nonspecific binding was evaluated with identical samples containing 100 µM isoproterenol, and was typically 15% of the total binding. In some experiments, displacement of ligand binding was carried out with the specific β₁-receptor antagonist, CGP20712A (Research Biochemicals International, Natick, MA) to identify the receptor subtype present on MDA-MB-231 cells. Scatchard determinations to identify changes in receptor number (B_{max}) or affinity (K_d) were carried out over a range of [¹²⁵I]iodopindolol concentrations from 0.02 to 1 nM.

Adenylyl cyclase activity. Cell membranes were prepared by the same procedure as for β-receptor binding, except that the buffer consisted of 250 mM sucrose, 1 mM EGTA, 10 mM Tris (pH 7.4). Aliquots of membrane preparation containing ≈20 µg protein were then incubated for 30 min at 30° C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM adenosine 5'-triphosphate, 10 mM MgCl₂, 1 mg bovine serum albumin, and a creatine phosphokinase-ATP-regenerating system consisting of 10 mM sodium phosphocreatine and 8 I.U. phosphocreatine kinase, and 10 µM GTP in a total volume of 250 µl. The enzymatic reaction was stopped by placing the samples in a 90-100° C water bath for 5 min, followed by sedimentation at 3000 × g for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay kits (Amersham Corp., Chicago, IL). Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay time period and was linear with membrane protein concentration; concentrations of cofactors were optimal and, in particular, the addition of higher concentrations of GTP produced no further augmentation of activity. In addition to evaluating basal activity, the maximal total activity of the adenylyl cyclase catalytic unit was evaluated with the response to 10 mM MnCl₂ (Chaudhry and Granneman, 1991).

The contributions of G-protein-linked processes to adenylyl cyclase were evaluated in two ways. First, to determine the net G-protein-linked response of adenylyl cyclase activity with maximal activation of all G-proteins, samples were prepared containing 10 mM NaF in the presence of GTP (Chaudhry and Granneman, 1991). Second, β -adrenoceptor-targeted effects mediated through the G-proteins were evaluated with 100 μ M isoproterenol in the presence of GTP. The concentrations of all the agents used here have been found previously to be optimal for effects on adenylyl cyclase and were confirmed in preliminary experiments (Chaudhry and Granneman, 1991; Navarro et al., 1991).

Data analysis. Data are presented as means and standard errors. For each study, treatment-related differences were first evaluated by a global ANOVA, incorporating all variables in a single test. For studies of adenylyl cyclase activity, multiple measurements were made from the same membrane preparation since several different stimulants compared; in that case, stimulant was considered a repeated measure. For studies of blockade of one drug by another, or of additive or synergistic effects, the combined effects were evaluated by two-factor ANOVA with the working hypothesis dependent upon a significant interaction between the two treatments. Where significant treatment effects were identified with the global test, individual differences between treatment groups were established with Fisher's Protected Least Significant Difference.

Scatchard plots were fitted by linear regression analysis and treatment-related differences were first compared by ANCOVA. Differences in maximal binding capacity (B_{\max}) and the equilibrium dissociation constant (K_d , the reciprocal of receptor affinity) were then evaluated using Fisher's Protected Least Significant Difference.

Significance for main treatment effects was assumed at $p < 0.05$ and interaction terms were considered significant at $p < 0.1$ (Snedecor and Cochran, 1967). For convenience, some data are presented as a percentage of control values but statistical significance was always assessed on the unmanipulated data. Where multiple time points are presented in the same graph, the control groups are given as a single value (100%), but statistical comparisons were conducted only with the time-matched group appropriate to each treatment.

RESULTS

MDA-MB-231 cells were in log-phase growth from 1 to 4 days after plating (Fig. 1). Over this span, DNA synthesis was maintained at a nearly constant rate and the number of cells, indicated by total DNA content, rose substantially. The increase in confluence between 1 and 4 days (more than double) was larger than the increase in DNA content (65%), indicating that cell enlargement was also occurring over this span. Drug treatments were initiated after one day in culture and were terminated at various times during log-phase growth, no later than four days in culture.

Addition of as little as 1 nM of isoproterenol to the medium produced immediate and robust inhibition of DNA synthesis (Fig. 2). The effect was maximal at 100 nM isoproterenol and was maintained throughout a 48h exposure. At the end of that period, isoproterenol-treated cells showed a significant reduction in the number of cells, assessed by DNA content. To demonstrate that the effects of isoproterenol were mediated through β -adrenoceptors stimulating the production of cAMP, a comparison was made with the membrane permeable cAMP analog, 8-Br-cAMP, and with the effects of the β -receptor antagonist, propranolol (Fig. 3). Isoproterenol and 8-Br-cAMP were equally effective toward DNA synthesis, and the effect of isoproterenol was completely blocked by propranolol. Propranolol by itself had no effect.

In developing tissues, glucocorticoid administration can sensitize cells to β -adrenoceptor agonists by inducing receptor formation and by enhancement of signaling components of the adenylyl cyclase cascade (Slotkin et al., 1994a). Accordingly, we examined whether dexamethasone enhances the ability of isoproterenol to inhibit DNA synthesis and to reduce the number of cells (Fig. 4). By itself, a 24h or 48h pretreatment with dexamethasone caused 20% inhibition of DNA synthesis and a significant reduction in cell number. When cells were pretreated for 48h with dexamethasone and then received a 2h challenge with isoproterenol, the inhibitory effects on DNA synthesis were less than additive: the net effect on DNA synthesis was no greater than that of isoproterenol alone, and the effect on DNA content was not distinguishable from that seen with just the dexamethasone pretreatment. However, when both

48h, the net effects on DNA synthesis and DNA content were augmented: decrements were greater than those achieved by either treatment alone.

In addition to measurements of DNA content, drug effects on the number of MDA-MB-231 cells were established by actual cell counts (Fig. 5). Sustained isoproterenol treatment reduced the total number of cells by over 20% and a comparable effect was seen for dexamethasone. Combined treatment with dexamethasone and isoproterenol had a comparably greater effect (30%), albeit not equivalent to the summation of the two individual effects. In order to maintain cAMP levels at the highest possible value, we also treated the cells with the phosphodiesterase inhibitor, theophylline, with or without isoproterenol (Fig. 5). Theophylline completely arrested mitosis, so that addition of isoproterenol had no further effect. The selectivity of the treatments towards mitosis were confirmed by examining cell morphology (Fig. 6). Isoproterenol and dexamethasone, alone or in combination, reduced the number of cells. Theophylline caused massive reductions in cell number but the remaining cells were correspondingly larger than in the control group, indicating that the treatment did not prevent postmitotic cell growth.

To determine whether the effects of isoproterenol are shared by all cancer cells expressing β -adrenoceptors, we compared the effects on MDA-MB-231 cells with those of rat C6 glioma cells (Fig. 7). In contrast to the human breast cancer cells, C6 cells showed neither inhibition of DNA synthesis nor a reduction in DNA content over comparable periods.

The maintenance of isoproterenol-induced inhibition of DNA synthesis in MDA-MB-231 cells over a 48h span of continuous treatment suggested that either agonist-induced receptor downregulation or desensitization were not present in these cells, or alternatively, that stimulation of only a small number of receptors was sufficient to inhibit mitosis. Receptor downregulation can be selective for different subtypes and accordingly, we first evaluated which subtype was present in MDA-MB-231 cells. Using the β_1 -selective antagonist, CGP20712A, we found that displacement of [125 I]iodopindolol involved a single class of sites displaying an IC₅₀ in the μ M range (Fig. 8). For contrast, we prepared cardiac cell membranes from one day old rats (Slotkin et al., 1994a), who display predominance of the β_1 -subtype (Slotkin et al., 1994b): in

this preparation, CGP20712A displayed two IC₅₀ values, one in the nM range corresponding to the major cardiac receptor population, and a minor component which, like the MDA-MB-231 cells, displayed an IC₅₀ in the μ M range. Accordingly, the subtype expressed by MDA-MB-231 cells is almost exclusively β_2 .

We next determined whether isoproterenol treatment of MDA-MB-231 cells causes β_2 -receptor downregulation and/or uncoupling of the receptors from their ability to stimulate adenylyl cyclase. In untreated cells, adenylyl cyclase activity declined over 50% during the span of log-phase replication (Fig. 9). However, the adenylyl cyclase response to isoproterenol fell by a significantly smaller proportion than did any of the other measures and the concentration of β -receptors was maintained at the same level throughout replication and growth. Relative to total cyclase catalytic activity (Mn^{2+}), the isoproterenol response actually increased over the course of culturing. After 1 day in culture, isoproterenol evoked $75 \pm 3\%$ of the total catalytic response exemplified by Mn^{2+} , whereas after 3-4 days in culture, the two stimulations were indistinguishable: isoproterenol evoked $97 \pm 3\%$ of the total response ($p < 0.0001$ compared to the proportion after 1 day in culture).

Despite the fact that isoproterenol-induced inhibition of DNA synthesis was maintained throughout a 48h drug exposure, receptor downregulation was apparent immediately upon introduction of the drug (Fig. 10). A concentration-dependent reduction in receptor binding was evident within 1h, with nearly complete downregulation by 24h. Receptor binding then remained at 5-10% of control values throughout 72h of exposure. Isoproterenol concentrations as low as 1 nM produced significant, albeit submaximal, reductions in receptor binding after 24h of exposure. Scatchard analysis confirmed that the loss of receptor binding reflected a decrease in the number of receptors as measured by maximal binding, rather than a change in receptor affinity as monitored by the K_d . In additional studies, we found that a 2h daily isoproterenol exposure was sufficient to cause full receptor downregulation. We treated cells for three days in succession, using 100 μ M isoproterenol for 2h each day, followed in each case by 22h without drug; 22h after the third day's exposure, receptor binding was only $6 \pm 1\%$ of control values

($n=6$, $p < 0.0001$). Similarly, even when we reduced the concentration to $1 \mu\text{M}$ with exposure for 2h per day over a two day span, receptor measurements made 22h after the last exposure still indicated robust downregulation ($8 \pm 1\%$ of control, $n=6$, $p < 0.0001$).

We also determined whether agonist-induced receptor downregulation was accompanied by loss of the adenylyl cyclase response to isoproterenol (Fig. 11). Treatment of cells with $1 \mu\text{M}$ isoproterenol for 2h, which caused approximately a 25% reduction in β -receptor binding, also elicited a comparable loss of the membrane response of adenylyl cyclase to isoproterenol. However, changes at the level of G-protein function were evident: basal enzyme activity, measured in the presence of GTP, also showed significant and immediate reductions, and the response to maximal G-protein activation by fluoride was impaired by a small amount. After a 48h exposure to isoproterenol, desensitization of the membrane adenylyl cyclase response to isoproterenol reached 75%, not quite as large as the degree of receptor downregulation; again, effects on basal adenylyl cyclase activity also were present, but were not as notable as the change in the β -receptor-mediated response. At no point did we note any decline in the expression or catalytic activity of adenylyl cyclase itself, as monitored by the effect of Mn^{2+} .

In light of the enhanced effect on cell replication of the combination of dexamethasone and isoproterenol treatment, we also examined their interaction at the levels of β -adrenoceptor binding and adenylyl cyclase activity. Pretreatment of cells with dexamethasone for 24h had no effect on β -receptor binding (Fig. 12, top panel). However, when the pretreatment was combined with a subsequent, 4h exposure to isoproterenol, it enhanced the downregulation caused by the receptor agonist. Dexamethasone had only small effects on adenylyl cyclase activity (Fig. 12, bottom panel). By itself, dexamethasone lowered the membrane response to isoproterenol by a few percent. When dexamethasone pretreatment was superimposed on short-term isoproterenol treatment of the cells, basal adenylyl cyclase activity was inhibited slightly less than with isoproterenol alone and the fluoride response was inhibited somewhat more. However, the agonist-induced desensitization of the specific response to isoproterenol was just as prominent after dexamethasone pretreatment as it was without pretreatment.

DISCUSSION

Among the epithelial, endocrine and secretory cancer cell lines that express β -adrenoceptors, MDA-MB-231 human breast cancer cells exhibit comparatively high concentrations (Vandewalle et al., 1990; Draoui et al., 1991; Marchetti et al., 1991; Re et al., 1992, 1996). The B_{\max} found here, 350 fmol/mg protein, exceeds the receptor concentration found in typical noradrenergic target tissues by over an order of magnitude (Thai et al., 1996) and is similar to the level found in brain regions enriched in noradrenergic projections. It is thus of critical interest that stimulation of these receptors leads to immediate inhibition of DNA synthesis and, with prolonged exposure, reductions in the total number of cancer cells. This effect was not shared by another cancer cell line, C6 rat glioma cells, that express a different β -receptor subtype at lower levels: whereas we found that MDA-MB-231 cells express β_2 -receptors exclusively, C6 cells express primarily the β_1 -subtype (Homburger et al., 1981).

Isoproterenol-induced inhibition of DNA synthesis in the breast cancer cell line exhibited the characteristic properties of β -receptor actions mediated through adenylyl cyclase: complete blockade by isoproterenol, and sharing of the effect by a membrane-permeable cAMP analog. What was unexpected, however, was the fact that isoproterenol's effect did not disappear with prolonged treatment, and in fact, was maintained at exactly the same level as the initial effect. Ordinarily, β -adrenoceptor agonists elicit downregulation and desensitization over a time frame of a few minutes to hours (Stiles, 1989), and therefore a loss of effect was expected here. The first hint of atypical regulation of β -receptor signaling was provided simply by monitoring adenylyl cyclase responses under control conditions. Over a course of several days in culture, adenylyl cyclase activity declined, accompanied by a loss of responsiveness to stimulants acting either at the level of β -receptors (isoproterenol), G-proteins (fluoride), or cyclase itself (Mn^{2+}). However, the enzymatic response to isoproterenol showed a smaller decline than for any other stimulant, that is, the β -adrenoceptor response actually increased relative to the total amount of catalytic activity. In fact, after 3-4 days in culture, isoproterenol elicited the maximal possible activation of adenylyl cyclase, since the activity was indistinguishable from that seen with

addition of Mn^{2+} . In addition, the response to isoproterenol, which selectively activates G_s , was significantly greater than that to fluoride, which causes activation of both stimulatory and inhibitory G-proteins. The profound response to isoproterenol is unusual, since in most tissues, isoproterenol is incapable of eliciting adenylyl cyclase activation equalling that of direct G-protein or cyclase stimulants (Navarro et al., 1991; Giannuzzi et al., 1995; Wagner et al., 1995; Zeiders et al., 1997). The number of β -receptors also kept pace with cell division and growth, so that the overall concentration of receptors remained unchanged throughout 4 days in culture, a period in which the number and size of cells increased substantially.

Given the maintenance of the ability of isoproterenol to inhibit DNA synthesis, we expected to see failure of receptor downregulation and/or desensitization, paralleling the situation during differentiation of normal cells possessing these receptors (Navarro et al., 1991; Giannuzzi et al., 1995; Zeiders et al., 1997). Surprisingly, isoproterenol caused immediate and robust receptor downregulation, accompanied by a parallel loss of the ability of receptor activation to stimulate adenylyl cyclase activity. Downregulation reached over 90% within 24h and the adenylyl cyclase response was desensitized by 75% throughout the period in which inhibition of DNA synthesis was maintained. These results thus indicate that downregulation and desensitization do occur, but that the ability of receptor stimulation to inhibit DNA synthesis and cell acquisition requires activation of only a very small number of receptors. This raises the possibility that there may be adaptations in the signaling pathway downstream from receptors, G-proteins and cyclase, that serve to maintain the net effect of receptor stimulation. In keeping with this view, we have found that, during brain development, the initial stimulation of β -receptors "programs" cAMP response elements so as to preserve or enhance the response of gene expression to adrenergic input (Wagner et al., 1994, 1995). If similar events occur in MDA-MB-231 cells, then adaptations of downstream elements may preserve the effects on cell replication in the face of receptor downregulation and desensitization. Accordingly, a logical next step is to look at transcription factors and genes targeted by β -receptor stimulation and their role in the antimitotic effect of isoproterenol.

From both the standpoints of mechanism and therapeutics, our findings of augmented effects with cotreatment of dexamethasone or theophylline are potentially important. Initially, we expected dexamethasone to enhance the response to isoproterenol because glucocorticoids induce the formation of β_2 -receptors in normal cells (Davies and Lefkowitz, 1984), especially during cell differentiation (Slotkin et al., 1994a); furthermore, these hormones also induce G-proteins and adenylyl cyclase in the fetus (Slotkin et al., 1994a). Dexamethasone treatment by itself inhibited DNA synthesis in MDA-MB-231 cells leading eventually to a reduction in cell number. In addition, when dexamethasone was combined with isoproterenol treatment, we saw even greater inhibition of DNA synthesis and loss of cells. However, when we examined the mechanism underlying the combined effect, we found that dexamethasone was not capable of preventing agonist-induced β -receptor downregulation or desensitization. Accordingly, the results again imply that actions downstream from the generation of cAMP are recruited to maintain the antimitotic response to receptor stimulation.

The second cotreatment that we examined was the phosphodiesterase inhibitor, theophylline. This drug alone had the greatest effect on mitosis: after several days in culture, the number of cells appeared to be no greater than that originally plated. Theophylline has a greater effect than isoproterenol because it interrupts the ability of phosphodiesterase to limit the rise of intracellular cAMP levels. However, in light of the findings for isoproterenol and dexamethasone, it is again possible that theophylline may influence gene expression downstream from cAMP generation, or alternatively, may act through other surface receptors (*e.g.* adenosine receptors) whose expression has not been explored in these cell lines.

Regardless of the ancillary mechanisms involved in β -adrenoceptor-mediated inhibition of mitosis in MDA-MB-231 breast cancer cells, the fact that inhibition does not disappear with receptor downregulation and desensitization raises the possibility for therapeutic strategies employing receptor agonists, alone or in combination with glucocorticoids and phosphodiesterase inhibitors. The cell line studied here, for example, is estrogen-insensitive and is thus nonresponsive to standard antiestrogen therapies. Furthermore, interventions operating at

the level of cell surface receptors, such as β -adrenoceptors, do not require penetration of drug to the interior of the cell, and thus would not be subject to loss of effect from induction of transporters in multidrug resistance. From a mechanistic standpoint, receptor input is "upstream" from genes, whose mutations lead to constitutive activation and cell proliferation (Mirossay et al., 1992; Moens et al., 1993; Wagner et al., 1994); receptor stimulation could thus limit the net effects of adverse mutations on cell cycle control by restricting expression of these genes. Given that desensitization effectively terminates the physiological effects of β -agonists in normal cells, therapeutic interventions based on receptor targeting should have only short-term side effects relative to the maintenance of effect in the target cell population; furthermore, the pharmacokinetics and toxicity of these agents are well-established. Neurotransmitter-based therapeutic strategies should be explored in an *in vivo* model to establish the potential utility of this general approach. If successful, screening of human cancers for the presence of β -adrenoceptors, or indeed, of other cell surface receptors, along with *in vitro* evaluation of the response of the cells to receptor agonists or antagonists, may establish new treatment strategies.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Cell replication and growth characteristics of MDA-MB-231 cells in culture. Data represent means and standard errors obtained from 40-60 determinations at each time point. For all experiments, treatments were initiated after one day in culture and were terminated no later than four days in culture.

Fig. 2. Effects of isoproterenol treatment on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 12-96 determinations for each concentration and time point. Isoproterenol was added for 2h or 48h, with inclusion of [^3H]thymidine for the final hour. ANOVA across all treatments appears at the top of each panel and asterisks denote individual treatments that differ significantly from the control.

Fig. 3. Effects of a 2h treatment with isoproterenol (Iso), 8-bromo-cAMP (8Br), or propranolol (Pro) treatment on DNA synthesis, presented as the percentage change from control values. Data represent means and standard errors obtained from 10-26 determinations for each concentration and time point. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the dagger denotes a significant difference between Pro + Iso and Iso alone. In addition, two-factor ANOVA for the effects of propranolol on the isoproterenol response indicated complete blockade ($p < 0.002$ for the main effect of isoproterenol, $p < 0.02$ for the main effect of propranolol, $p < 0.05$ for the interaction of the two treatments). None of the treatments produced a significant change in DNA content (data not shown).

Fig. 4. Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on DNA synthesis and content, presented as the percentage change from control values. Data represent means and standard errors obtained from 11-54 determinations for each treatment. ANOVA across all treatments appears at the top of each panel; asterisks denote individual treatments that differ significantly from the control and the daggers denote significant differences between Dex 48h + Iso 48h, and the corresponding treatments with Dex or Iso alone. In

addition, for DNA synthesis, two-factor ANOVA (dexamethasone 48h \times isoproterenol 2h) indicates significant main effects of dexamethasone ($p < 0.0005$) and isoproterenol ($p < 0.0001$) but no interaction between the two treatments; with 48h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each) as well as a significant interaction of dexamethasone \times isoproterenol ($p < 0.002$). For DNA content, two-factor ANOVA (dexamethasone 48h \times isoproterenol 2h) indicates a significant main effect of dexamethasone ($p < 0.009$) but no effect of isoproterenol; with 48h of both dexamethasone and isoproterenol treatment, there were significant main effects of both treatments ($p < 0.0001$ for each).

Fig. 5. Effects of a 72h treatment with isoproterenol (Iso), dexamethasone (Dex), or theophylline (Theo) on cell number, presented as the percentage change from control values. Data represent means and standard errors obtained from 10-22 determinations for each treatment. ANOVA across all treatments appears at the top of each panel. Asterisks denote individual treatments that differ significantly from the control; the dagger denotes a significant difference between Dex + Iso and either treatment alone; § denotes significant differences between theophylline with or without isoproterenol, as compared to all other treatments. In addition, two-factor ANOVA across the dexamethasone and isoproterenol treatments indicates significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of dexamethasone \times isoproterenol ($p < 0.03$). Across the theophylline and isoproterenol groups, there were significant main effects of each treatment alone ($p < 0.0001$) as well as a significant interaction of theophylline \times isoproterenol ($p < 0.0001$).

Fig. 6. Phase-contrast microscopic appearance of MDA-MB-231 cells after a 72h treatment with isoproterenol, dexamethasone, or theophylline. Scale bar appears in the upper left panel.

Fig. 7. Effects of 10 μ M isoproterenol (Iso) on DNA synthesis and content in rat C6 glioma cells, presented as the percentage change from control values. Data represent means and standard errors obtained from 11-30 determinations for each time point. ANOVA across all treatments appears within each panel.

Fig. 8. Binding of [125 I]iodopindolol to β -adrenoceptors in cell membranes prepared from MDA-MB-231 cells and from one day old rat heart, and its displacement by the β_1 -specific antagonist, CGP20712A. Each data point represents an individual determination, shown as the percentage of values obtained in the absence of displacer. The [125 I]iodopindolol concentration was 67 pM.

Fig. 9. Adenylyl cyclase activity and β -adrenoceptor binding in MDA-MB-231 cells during cell replication and growth in culture. Cells were cultured for the indicated time periods and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μ M isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+} . Data represent means and standard errors obtained from 6-24 determinations for each measurement at each time point. ANOVA across both time points and all stimulants appears within the panel and asterisks denote measures for which the values after 3-4 days in culture are significantly lower than the initial values. In addition, the cyclase response to isoproterenol declines less than the response to other stimulants ($p < 0.0001$).

Fig. 10. β -Adrenoceptor downregulation caused by isoproterenol treatment. Data represent means and standard errors obtained from the number of determinations shown in parentheses, determined as the percentage change from control values. In the top and middle panels, ANOVA across all time points or treatments appears at the top and asterisks denote individual values that differ significantly from the control. In the bottom panel, ANCOVA appears for the overall differences between Scatchard plots in control and isoproterenol-treated (Iso) cells.

Fig. 11. Desensitization of adenylyl cyclase evoked by exposure to 1 μ M isoproterenol. Cells were treated with isoproterenol for the indicated time period and then membranes were isolated and enzyme activity determined under basal conditions or in the presence of 100 μ M isoproterenol (Iso), 10 mM fluoride (F^-) or 10 mM Mn^{2+} . Data represent means and standard errors obtained from 6-12 determinations at each time point, determined as the percentage change from control values. ANOVA across all stimulants and both time points appears at the top and asterisks denote individual values that differ significantly from the control. Two-factor

ANOVAs (treatment \times time) were also assessed for each variable. Across both time points, basal activity was significantly reduced ($p < 0.0001$ for the main effect of isoproterenol), the response to isoproterenol was reduced in a time-dependent fashion ($p < 0.0001$ for the main effect, $p < 0.0001$ for the treatment \times time interaction), the response to fluoride was reduced ($p < 0.0001$ for the main effect), and the response to Mn^{2+} was unaffected.

Fig. 12. Effects of dexamethasone (Dex) alone or in combination with isoproterenol (Iso), on β -adrenoceptor binding (top) and adenylyl cyclase activity (bottom). Cells were pretreated with 10 μ M dexamethasone for 24h (top) or 48h (bottom) and were then exposed to 1 μ M isoproterenol for 4h. For adenylyl cyclase, enzyme activity was determined under basal conditions or in the presence of 100 μ M isoproterenol (Iso), 10 mM fluoride (F) or 10 mM Mn^{2+} . Data represent means and standard errors obtained from 6-12 determinations for each treatment. For receptor binding, ANOVA across all treatments appears at the top; for adenylyl cyclase, ANOVA across all treatments and *in vitro* stimulants appears at the top, and lower order ANOVAs are shown for *in vitro* stimulant with each cluster of bars. Asterisks denote individual treatments that differ significantly from the control and the daggers denotes significant difference of combined treatment from the effects of dexamethasone or isoproterenol alone.

Index terms: adenylyl cyclase

β -adrenoceptors

cell replication

cyclic AMP

dexamethasone

DNA synthesis

glucocorticoids

Fig. 1

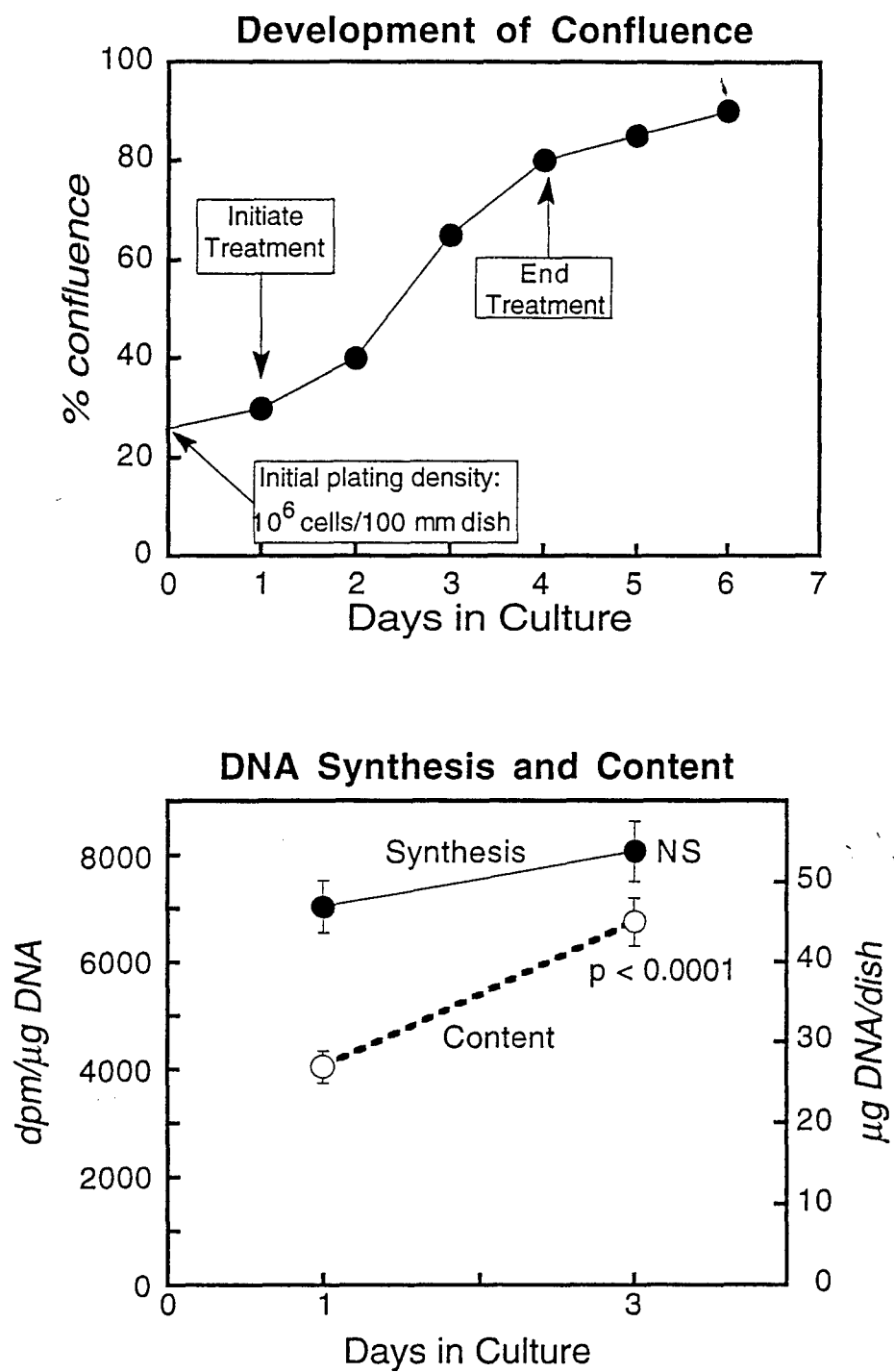


Fig. 2

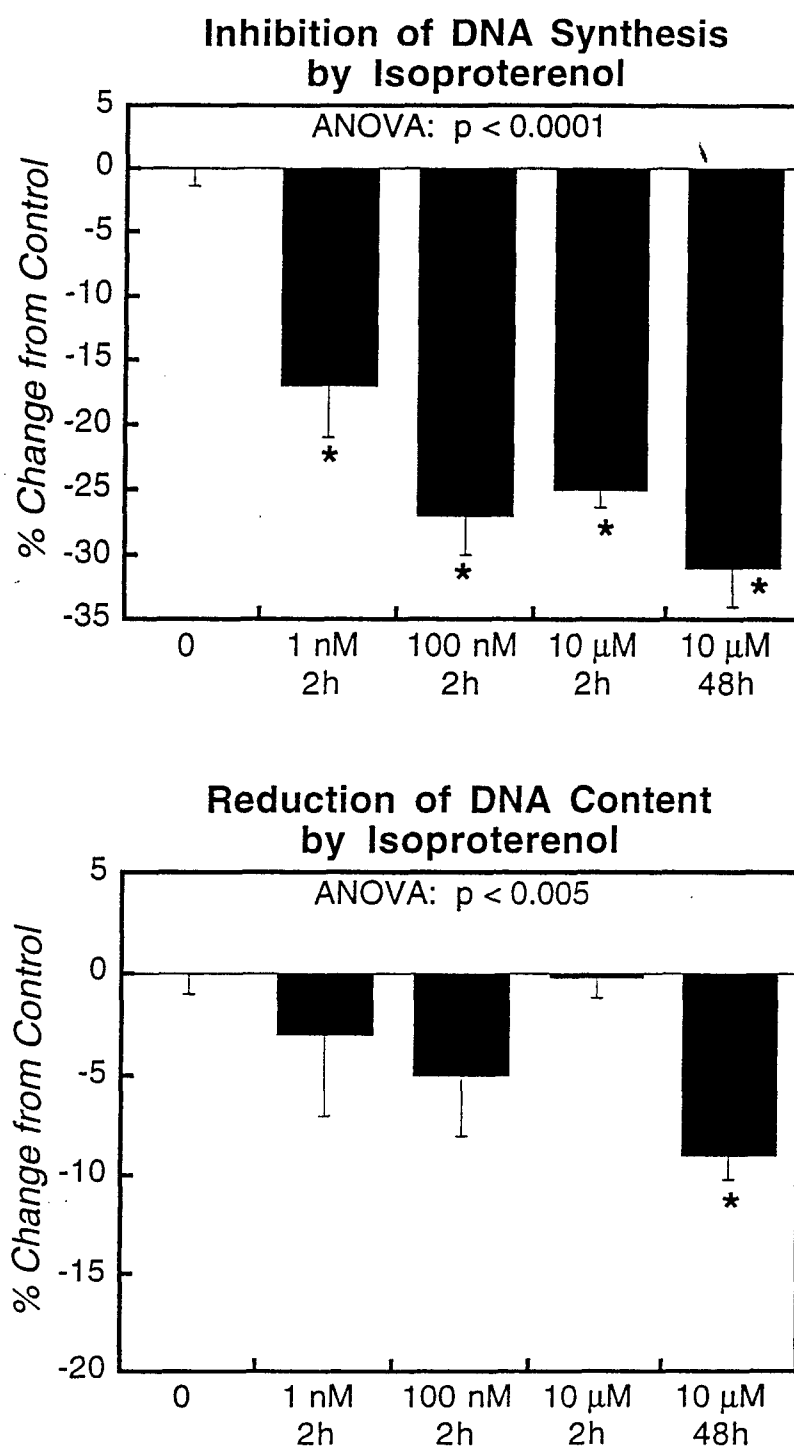


Fig. 3

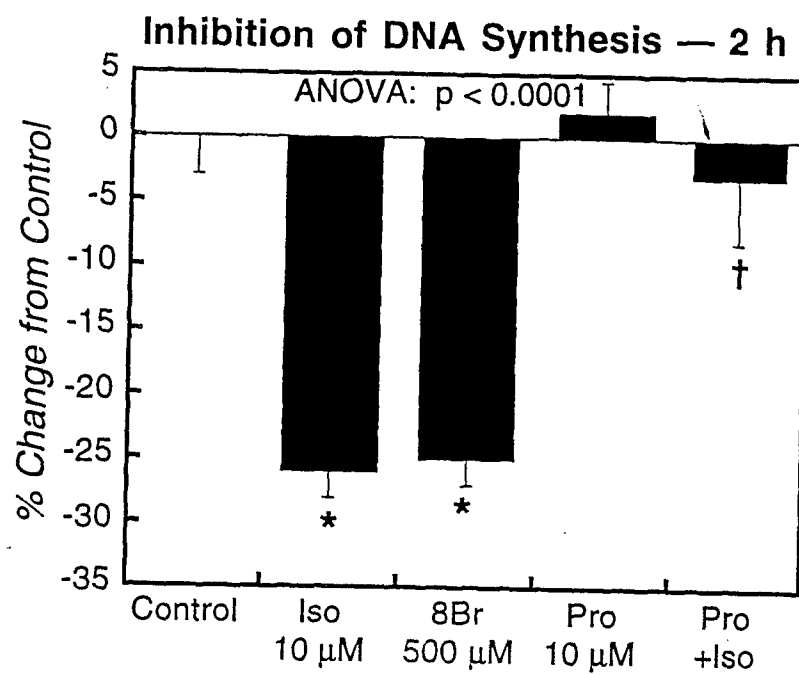


Fig. 4

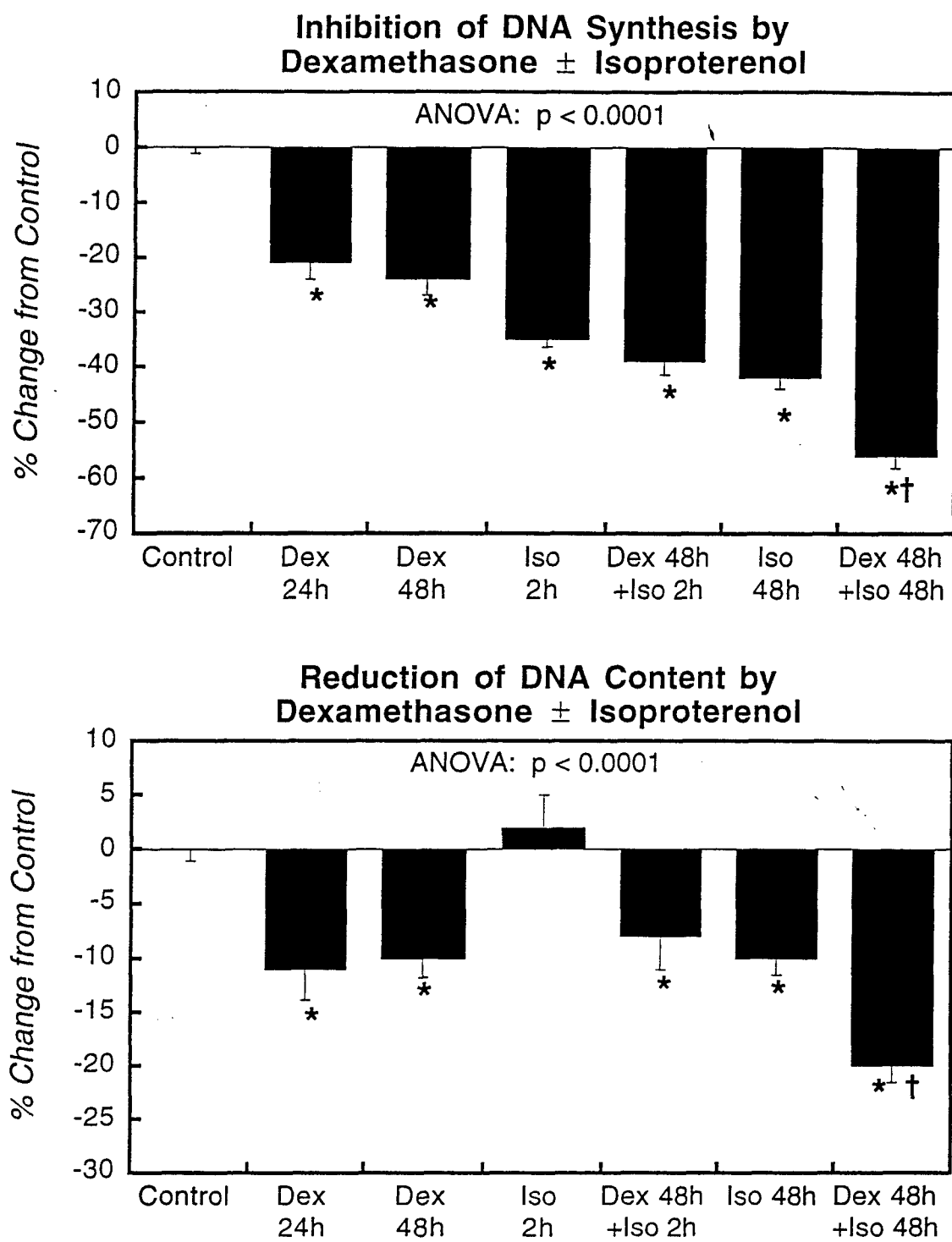


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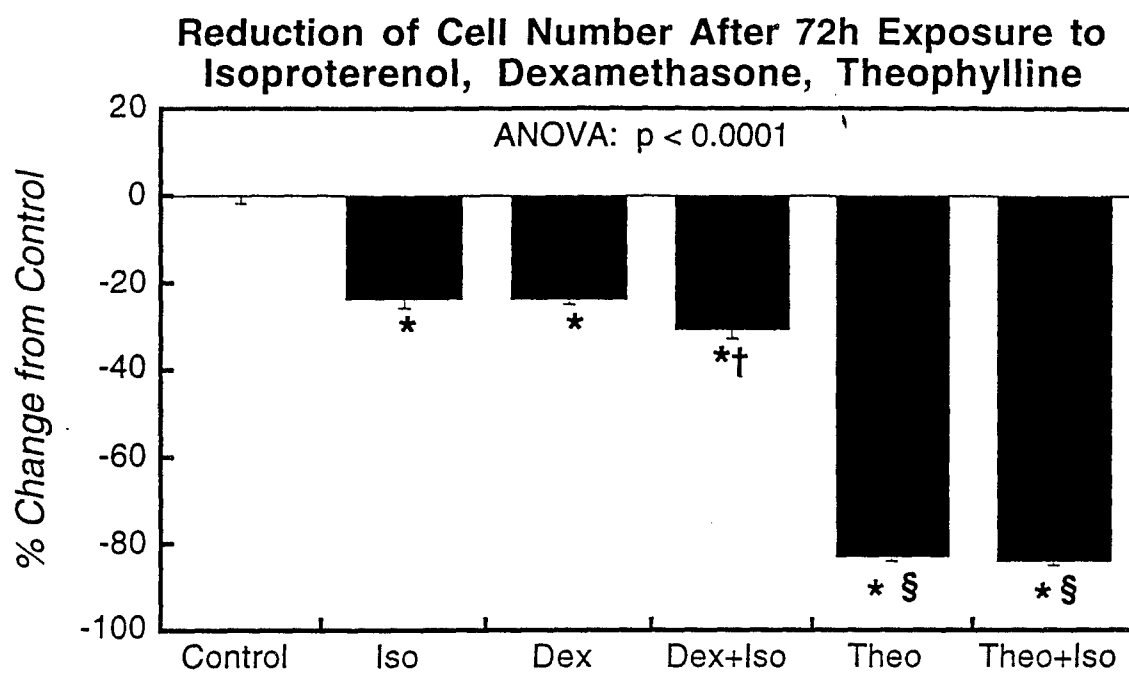
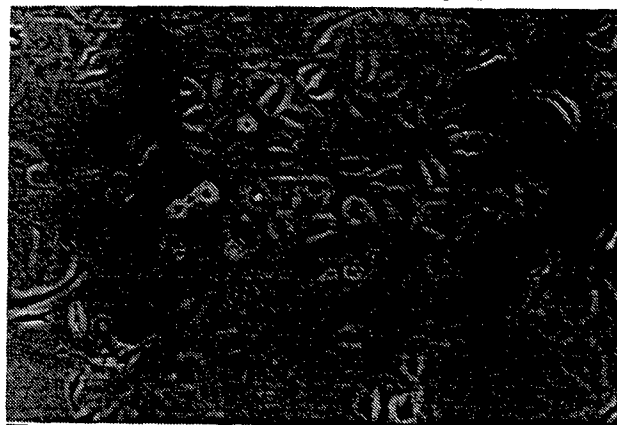


Fig. 6

CONTROL



ISOPROTERENOL



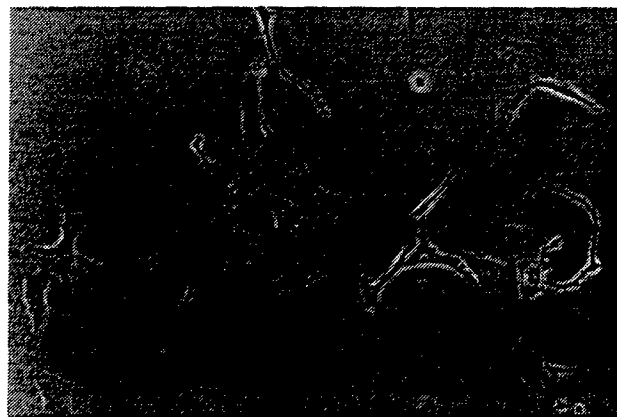
DEXAMETHASONE



**DEXAMETHASONE
+ ISOPROTERENOL**



THEOPHYLLINE



**THEOPHYLLINE
+ ISOPROTERENOL**

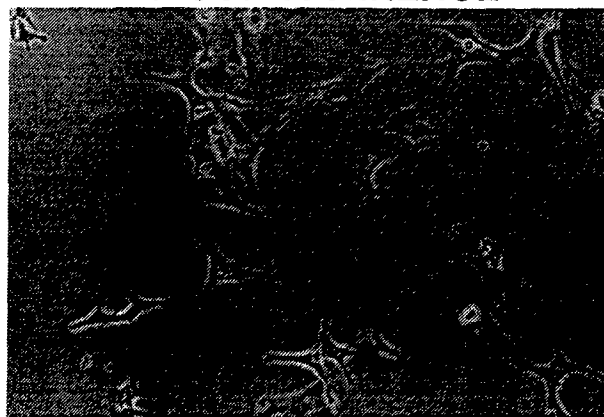


Fig. 7

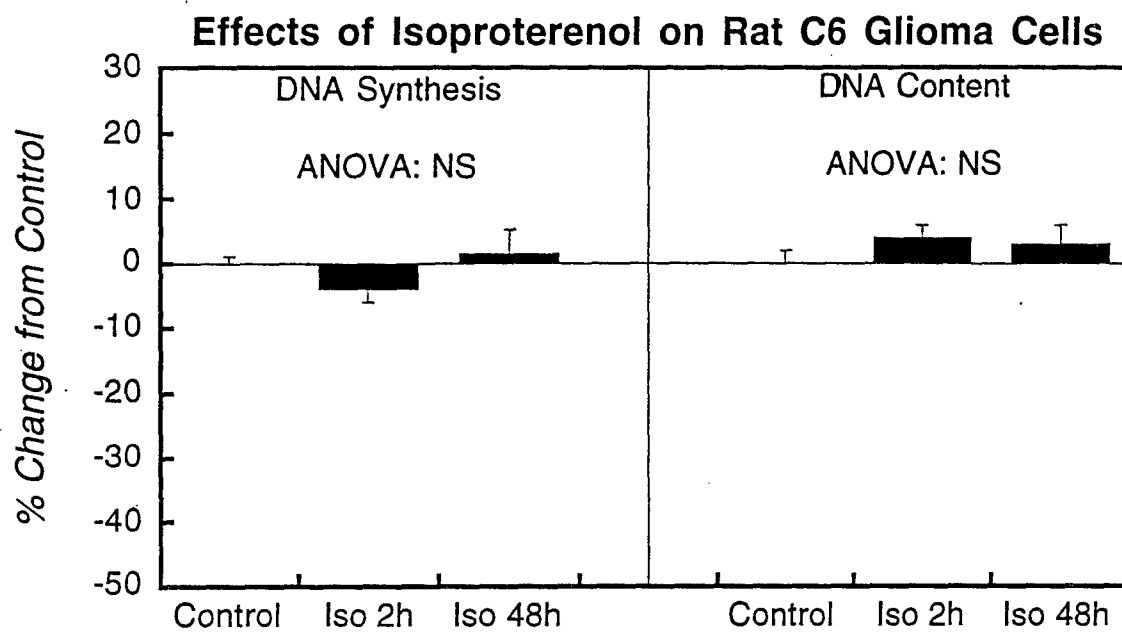


Fig. 8

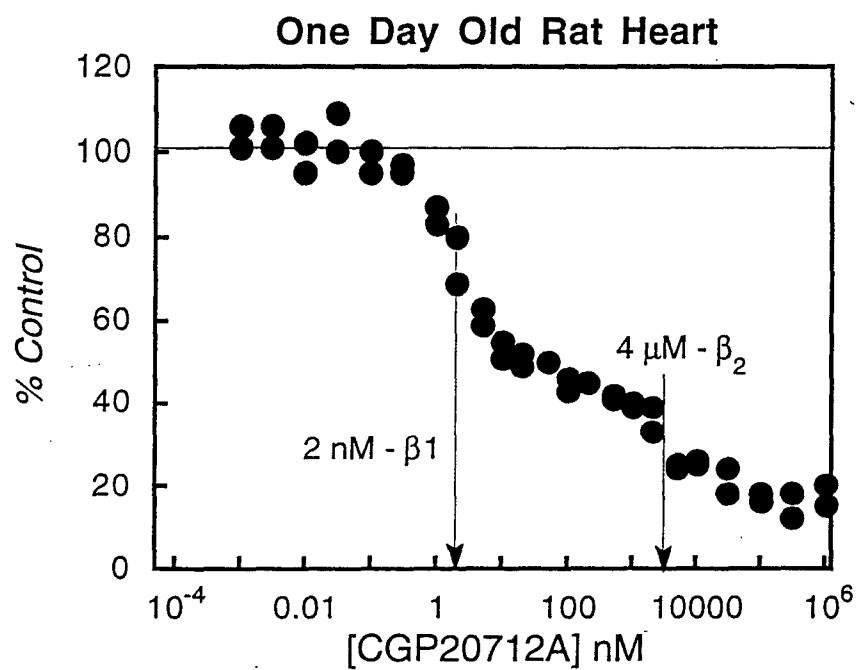
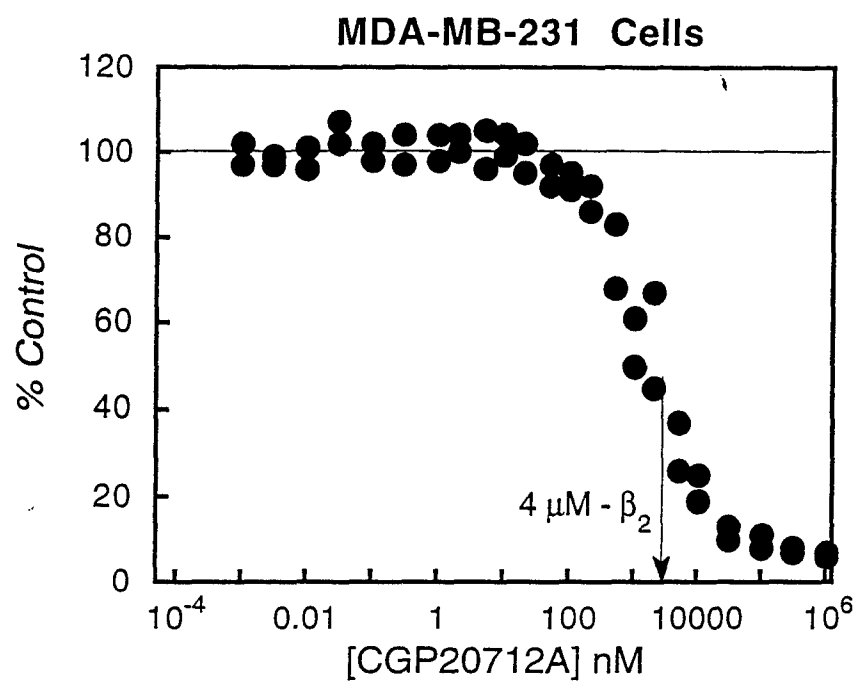


Fig. 9

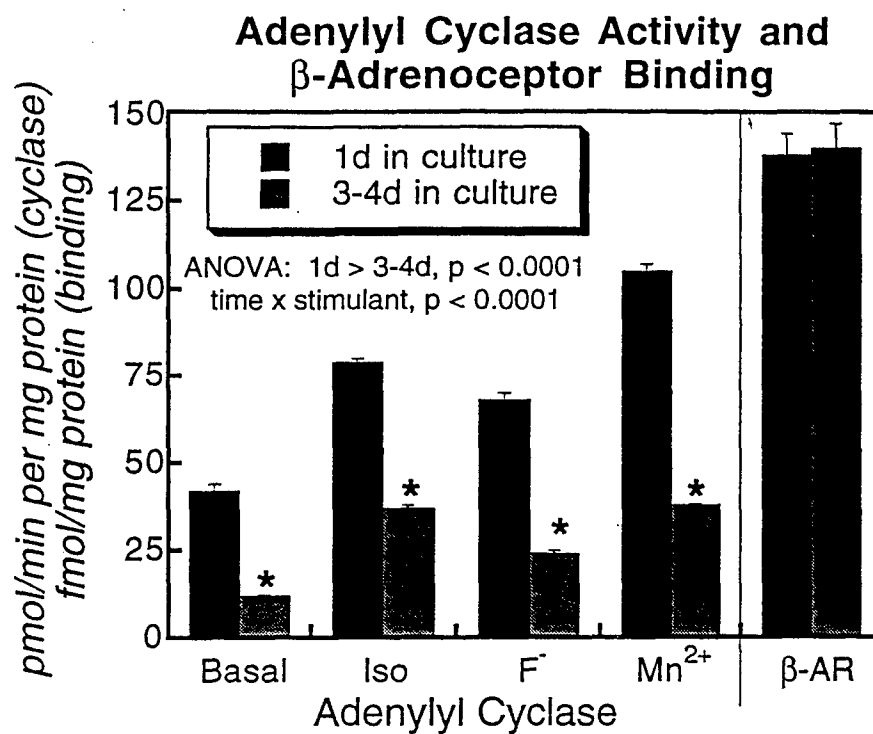
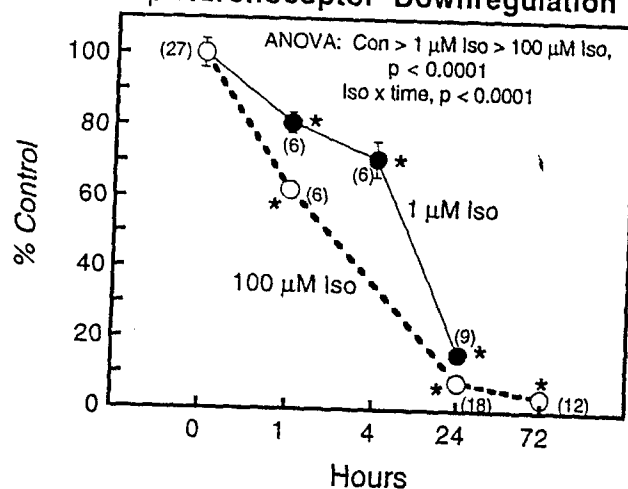
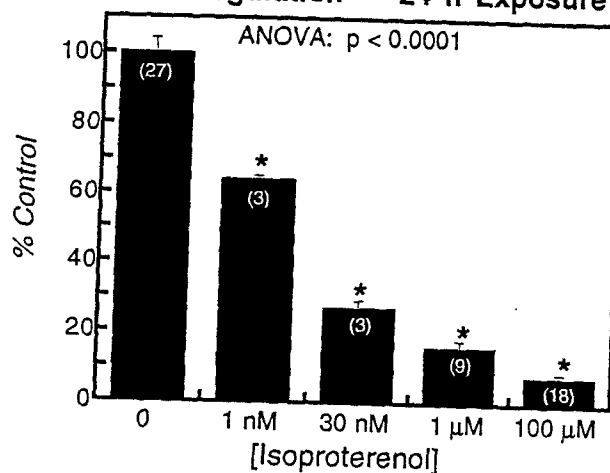


Fig. 10

Time Course of Isoproterenol-Induced β -Adrenoceptor Downregulation



Concentration-Response Relationship for Isoproterenol-Induced β -Adrenoceptor Downregulation — 24 h Exposure



β -Receptor Binding

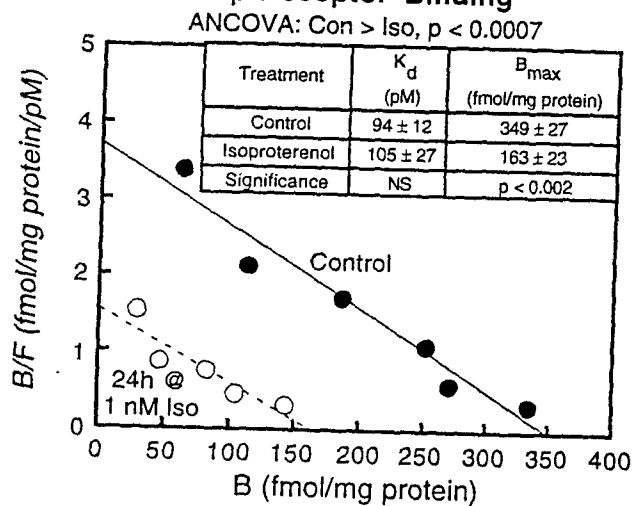


Fig. 11

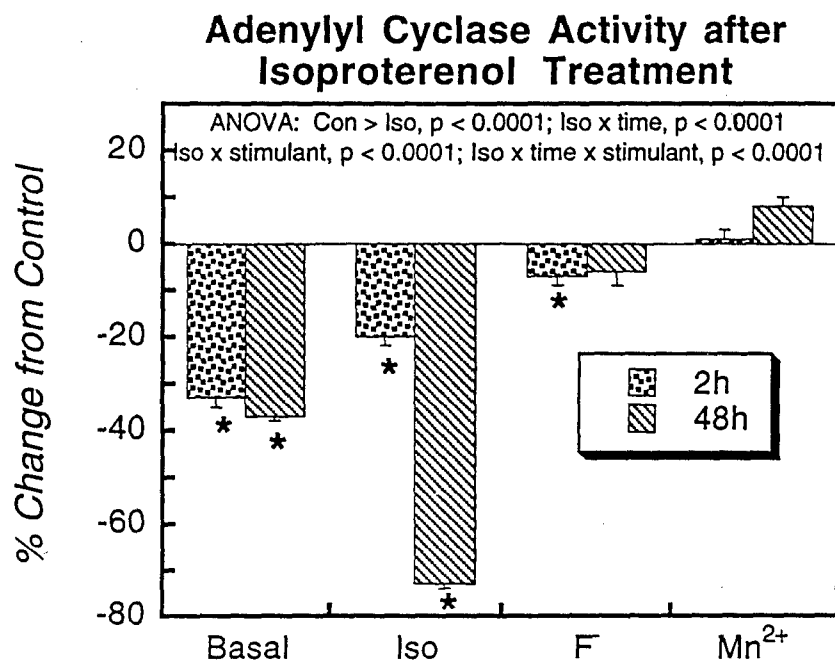
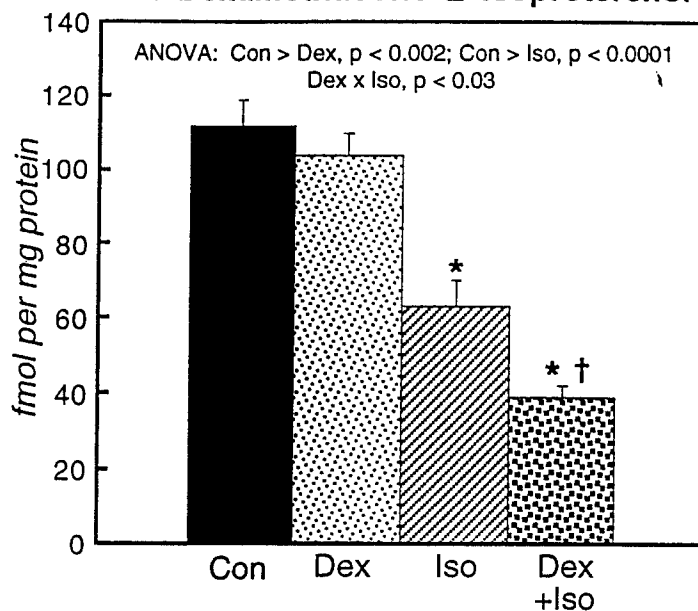


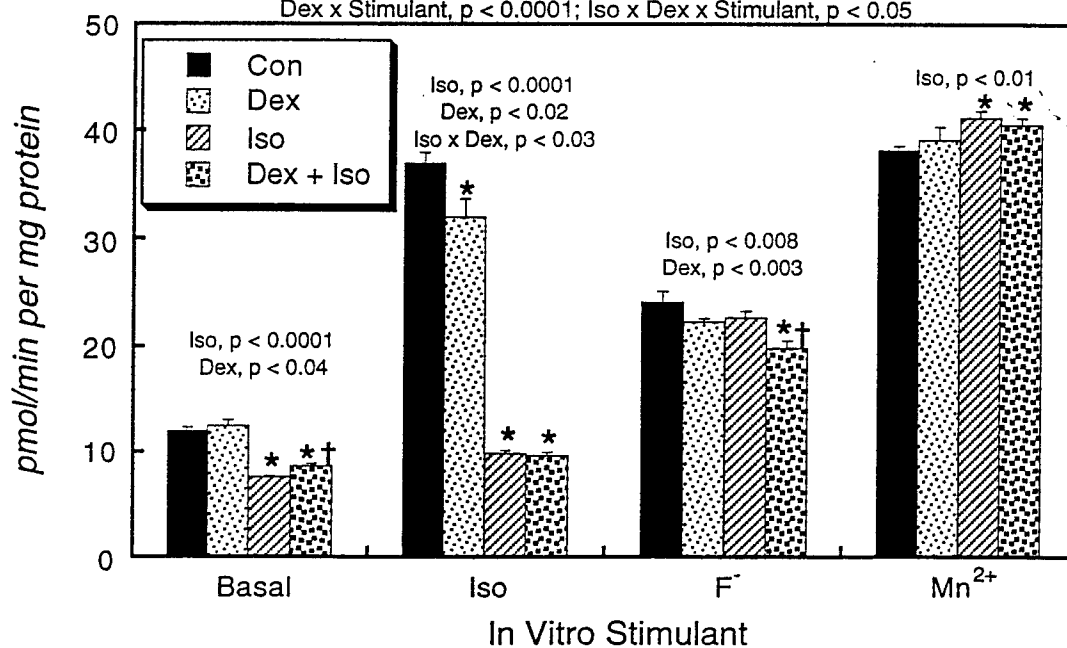
Fig. 12

β -Adrenoceptor Binding After Treatment with Dexamethasone \pm Isoproterenol



Adenylyl Cyclase Activity After 48 h Treatment with Dexamethasone \pm Isoproterenol

ANOVA: Con > Iso, $p < 0.0001$; Iso x Stimulant, $p < 0.0001$
Dex x Stimulant, $p < 0.0001$; Iso x Dex x Stimulant, $p < 0.05$





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